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Polypeptide Synthesis by mRNA Isolated from a Variety of Barley and its *Virescens* Mutant

by

© Elizabeth A. Weretilnyk

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Ph.D

IN

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Department of Plant Science

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I would like to dedicate this thesis to

the memory of my father

Abstract

As the mutation responsible for *virescens* resides in the nuclear genome, attention was turned towards the analysis of poly(A) RNA directed polypeptide products since this fraction of RNA is generally equated with nuclear-encoded products. Isolation and subsequent *in vitro* translation of poly(A) RNA was carried out using both wheat germ and reticulocyte lysate cell-free protein synthesizing systems. *In vitro* translation of the isolated poly(A) RNA demonstrated that the mutant is well-endowed with highly-translatable mRNA. Electrophoretic separation of the translation products directed by poly(A) RNA isolated from normal (N) and mutant (M) seedlings indicated that polypeptides of both lines changed from 4 to 6 days (d) and that the most variability between N and M products occurred at 4 d of growth and appeared to be quantitative rather than qualitative. However, even these subtle differences were no longer apparent by 5 d. A pronounced qualitative variation which persisted was between translation products of the two cell-free sources reflecting the differing degrees of selectivity and translational ability of the wheat germ and reticulocyte lysate systems.

Comparison of polysomal profiles from preparations of N and M apical leaf segments showed that polysome aggregation in response to light occurred for both N and M. Messages associated with M profiles encode a number of polypeptides. However, the message associated with the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is not well-represented among 4 d M polysomal mRNA. This provides an explanation for the extremely low levels of Rubisco detected at 4 d. In contrast, *in vitro* translation products directed by mRNA for the precursor for the small subunit (pSSU) can be readily detected among poly(A) RNA products and these messages are associated with polysomes. The failure of the M to accummulate Rubisco to wild-type levels is probably a function of the reduced complement of LSU. Furthermore, M polysomal profiles are readily distinguished from those of the N by the reduced contribution of a peak which migrates at a position generally occupied by 70S ribosomes. This observation substantiates an earlier suggestion that the M is deficient in 70S ribosomes. This deficiency, not unlike that of the pigments, appears to dissipate over time.

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Table of Contents

Chapter — Page

I. Introduction 1

II. Protein Synthesis in Leaves 4

A. Chloroplast Development 4

B. Plant Genome Organization 5

Chloroplast genome analysis 6

Nuclear genome analysis 8

C. Regulation of Gene Expression 13

Transcriptional Regulation 13

Post-transcriptional Regulation 15

D. Control and Co-ordination of Protein Synthesis 19

III. Materials and Methods 20

A. Plant Material 20

B. Materials 21

C. Isolation of RNA 21

Extraction of Total RNA 21

Poly(A) RNA Separation 22

Quantification, Purity and Storage of RNA 23

D. Cell-Free Protein Synthesis 24

Wheat Germ Cell-Free System 24

Preparation of Lysate 24

Protein Synthesis In vitro 25

Rabbit Reticulocyte Lysate Cell-Free System 25

Incorporation Measurements 25

Analysis of Products 26

Electrophoretic Separation of Products 26

Immunoprecipitation of In vitro *Translation Products* 27

E. Polyribosome Analysis 28
Polysome Extraction 28
Polysome Gradients 29
Polysomal RNA Extraction 29
F. Total Ribonuclease Activity 30
IV. Results and Discussion 31
A. Characterization of Plant Material 31
B. Poly(A) RNA Analysis 36
Poly(A) RNA Yields 36
In vitro Translatability of Poly(A) RNA 45
Translation of Poly(A) RNA 47
Translation Products in a Wheat Germ System 48
Translation Products Synthesized in a Reticulocyte Lysate System 49
C. Polysome Profile Analysis 50
Assessment of Translational Capacity 50
RNA Analysis 58
V. Conclusions 82
Bibliography 90

List of Tables

Table		Page
I.	Poly(A) RNA yields from apical leaf segments of N and M barley seedlings grown under continuous light and under etiolated conditions.	38
II.	Total ribonuclease activity in apical leaf segments of 4 and 6 d N and M barley seedlings grown under continuous illumination.	38
III.	Translational activity of poly(A) RNA isolated fom apical leaf segments of N and M barley seedlings.	46
IV.	Analysis of polyribosome distribution in 4 and 6 d N and M barley apical leaf segments.	52

List of Figures

Figure — Page

1. Dry weight determinations of apical leaf segments expressed as a % of fresh weight measurements. 32

2. Chl a and b accumulation in apical laminae segments of N and M barley seedlings grown under continuous illumination. 33

3. Total chl accumulation in apical laminae segments of N and M barley seedlings grown under continuous illumination. 34

4. The ratio of chl a to chl b in apical laminae segments of N and M barley seedlings grown under continuous illumination. 35

5. Fluorogram of [^{35}S]-methionine labelled wheat germ translation products of poly(A) RNA isolated from 4 d N and M barley apical leaf segments of light-grown and etiolated seedlings. 40

6. Comparison of [^{35}S]-methionine labelled wheat germ translation products directed by poly(A) RNA isolated from light-grown 4, 5 and 6 d N and M barley apical leaf segments. 42

7. Comparison of [^{35}S]-methionine labelled reticulocyte lysate translation products directed by poly(A) RNA isolated from light-grown 4 and 6 d N and M barley apical leaf segments. 44

8. Sucrose gradient profiles of ribosomal extracts of 4 d N and M barley apical leaf segments prepared from seedlings grown under continuous illumination. 53

9. Sucrose gradient profiles of ribosomal extracts of 6 d N and M barley apical leaf segments prepared from seedlings grown under continuous illumination. 54

10. Sucrose gradient profiles of ribosomal extracts of 4 d N and M barley apical leaf segments prepared from etiolated seedlings. 55

11. Ribosomal extracts of 4 d etiolated N and M barley seedlings prepared to highlight the position of 70S ribosomes. 57

12. *In vitro* translatability of RNA extracted from 4 d N and M polysomal gradients. 59

13. *In vitro* translation products directed by total RNA isolated from fractions of 4 d N polysomal gradients. 62

14. *In vitro* translation products directed by total RNA isolated from fractions of 4 d M polysomal gradients. 64

15. Fluorogram of reticulocyte lysate translation products of poly(A) RNA and polysomal RNA from 4 d N and M light-grown seedlings. 66

16. Fluorogram of wheat germ translation products of poly(A) RNA and polysomal RNA from 4 d N and M light-grown seedlings. 68

17. Fluorogram of [^{35}S]-methionine labelled reticulocyte lysate assay products directed by polysomal RNA isolated from 4 d N and M light-grown and etiolated seedlings. ..71

18. Fluorogram of [^{35}S]-methionine labelled wheat germ assay products directed by polysomal RNA isolated from 4 d N and M light-grown and etiolated seedlings. 73

19. Immunoprecipitation of pSSU of Rubisco from wheat germ translation products using poly(A) RNA and total polysomal RNA isolated from 4 d N light-grown seedlings. 77

20. Immunoprecipitation of pSSU of Rubisco from wheat germ translation products using poly(A) RNA and total polysomal RNA isolated from 4 d M light-grown seedlings. 79

21. Immunoprecipitation of LSU and pSSU of Rubisco from reticulocyte translation products using poly(A) RNA and total polysomal RNA from 4 d N and M light-grown seedlings. 81

List of Abbreviations

AMP	adenosine monophosphate
ATP	adenosine triphosphate
cDNA	complementary DNA
CF_0 or CF_1	coupling factors 0 or 1
chl	chlorophyll
CP1	P-700-chlorophyll a-protein
cpDNA	chloroplast DNA
d	day
D	dalton
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EF	elongation factor
EGTA	ethyleneglycol-bis(β-aminoethyl ether)-N,N'tetraacetate
GTP	guanosine triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
kD	kilodalton
LHCP	light harvesting chlorophyll a/b protein
LSU	large subunit of Rubisco
M	*virescens* mutant of barley cv. Gateway
mRNA	messenger ribonucleic acid
MW	molecular weight
N	wildtype barley cv. Gateway
NAD	nicotinamide-adenine-dinucleotide
NADP	nicotinamide-adenine-dinucleotide phosphate
OD	optical density
oligo d(T)	oligothymidylic acid
PAGE	polyacrylamide gel electrophoresis
PAR	photosynthetically active radiation
PEP	phosphoenolpyruvate
poly(A)	polyadenylic acid
PSI or II	photosystem I or II
psbA	gene for 32 kD atrazine-binding polypeptide
psbD	gene for 'D2', a 32 kD polypeptide
pSSU	precursor small subunit of Rubisco
rbcL	gene for LSU-Rubisco
rbcS	gene for SSU-Rubisco
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulfate
SE	standard error
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
UV	ultraviolet

I. Introduction

Classically, two main approaches have been used to study the development of chloroplast structure and function. The first approach has involved the use of etiolated plants and analysis of the progressive stages of de-etiolation which occur as plants are exposed to light of varying intensity and/or wavelength. Although the sequential steps of greening as demonstrated by the use of etiolated plants has contributed much to our understanding of photomorphogenesis, a second approach involving the use of plants arrested or delayed in chloroplast biogenesis, particularly mutants, has also provided important tools towards our knowledge of this subject.

Perhaps the most desirable mutant for a greening study would be one that displays a complete block in a single essential process required for plastid formation. However, as mutations of this nature are generally lethal, maintenance of these stocks often proves to be very laborious. Conditional lethal mutants are occasionally found and provide the advantage that plants can be encouraged to mature under permissive environmental conditions. A third class of mutants is comprised of plants for which the mutation proves to be non-lethal. Often these plants are distinguished by their phenotypically distinctive features as in the case of pigment-deficient plants. To their disadvantage, however, mutants of this genre often display pleiotropic effects and resolution of the actual lesion can prove to be an extremely difficult task.

Research conducted in this laboratory has been directed toward characterizing a mutant of barley which is best described by the third category. Initial studies demonstrated that young seedlings are deficient in chlorophylls a and b when compared to normal seedlings of its varietal origin (cv. Gateway) and that this deficiency became less marked as seedlings matured (127). The ability to recover near normal levels of chlorophyll within 2 weeks of germination established this pale-green mutant to be a *virescens* type. Inheritance studies carried out by Walker *et al.* (222) and Stephansen and Zalik (196) provided evidence that the mutation, designated *yellow-viable-2* (yv_2), involves a single, recessive nuclear gene and assigned it to

chromosome 1.

Other anomalous properties of these seedlings have been studied. Accumulation of pigments is sensitive to both temperature and light intensity (142). Various carotenoids are found in reduced levels in the M as compared to the N (142,202). The seedlings also possess elevated levels of free amino acids, most notably serine (127). With respect to plastid ultrastructure, chloroplasts of 4-day old seedlings exhibit only a very rudimentary lamellar system (106). However, 6 and 8-day old seedlings show a progressive increase in thylakoid membrane development, particularly evident in the formation of well-developed grana stacks (96,106). Under high light intensity a highly vesicular plastid is formed and many plastoglobuli accumulate (106). In dark-grown seedlings the prolamellar body, a distinctive feature of etioplasts (121), is not formed (107). The measurement of photochemical activities associated with photosystem II (PSII) (115,116) and photosystem I (PSI) (115) reveals that neither system is specifically altered by the mutation. However, fluorescence studies carried out on the mutant (116) suggest that some of the light-harvesting chlorophyll complex may not be associated with PS II reaction centers. Furthermore, analysis of photochemical activity and plastid membrane proteins (115) suggests that the transition of proplastid to chloroplast may proceed in a manner analogous to that of the normal, albeit at a substantially reduced rate. Similarly, a markedly reduced rate of accumulation of the prominent soluble protein of the chloroplast stroma, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), has also been reported (12). These studies, which have explored a diverse selection of metabolic processes, suggest that many, if not all facets of chloroplast development in these seedlings are affected by a single, nuclear gene mutation.

As the temporal lag in chloroplast development has been attributed to a nuclear source, the attention of this study was directed initially towards the characterization of the polyadenylated fraction of mRNA in developing seedlings. This study entailed the tailoring of methods that would maximize both yield and translatability of the isolated RNA. The well-documented developmental disparity between the mutant and its wild-type, particularly at

4 d, suggested that quantitative and, most probably, qualitative differences might be evident among populations of translatable mRNA. However, development is not merely a function of transcription. Consequently, the second objective of this study was an attempt to assess the translational capacity of mutant seedlings. The perspectives chosen to address this rather broad question involved evaluating the *in vitro* translatability of mRNA isolated from mutant seedlings in addition to determining the relative proportion of ribosomes as polysomes engaged in protein synthesis.

II. Protein Synthesis in Leaves

A. Chloroplast Development

A feature unique to plant cells is the possession of a class of organelles called plastids. The most extensively studied plastid type is the chloroplast, the plastid distinguished by the presence of chlorophyll.

In greening studies of angiosperms, the sequence of chloroplast development has been primarily studied using etiolated plants (121). In the case of these dark-grown plants, the prevailing form of plastid is either a proplastid, a largely undifferentiated organelle, or alternatively, an etioplast which is the somewhat differentiated plastid characteristic of etiolated plants (110,121,214). Although the etioplast has not been found in the developmental sequence of plastids that have not been subject to etiolation (110,121), exposure of either proplastid or etioplast to light leads to their rapid transformation into chloroplasts (110,214). Chloroplast "biogenesis" is a fundamental aspect of greening and, as a consequence, leaf development.

The transformation from proplastid or etioplast to chloroplast is accompanied by changes in plastid size, internal membrane structure and biosynthetic functions (110). Exposure of an etiolated leaf to light leads to a rapid disintegration of the prolamellar body and chlorophyll accumulation begins (110). Concurrent with these events is the formation of stroma and grana lamellae as the sequential development of PSI followed by PSII becomes evident (34,122). In addition to the proteins required for thylakoid membrane construction and electron transport components, other proteins are required for the Calvin cycle enzymes, photophosphorylation, photorespiration, lipid biosynthesis, nitrate metabolism, nucleic acid synthesis, protein synthesis and the many other activities associated with this organelle. It is clear that the development of a chloroplast in response to light is accompanied by substantial quantitative and many qualitative changes in plastid stromal and thylakoid proteins. Many studies have been conducted to determine the subcellular site of origin of chloroplast proteins

and to elucidate the nature of the controls that may serve to regulate chloroplast development in leaves.

B. Plant Genome Organization

There are essentially three genetic systems in plants. The most prominent system is under nuclear control. Reports published in the 1960s established that chloroplasts contain DNA and ribosomes, DNA polymerase, RNA polymerase and other factors required for nucleic acid and protein synthesis (110). Thus the chloroplast was shown to possess its own genetic system as has been demonstrated for the mitochondrion.

As the chloroplast contains its own machinery to carry out transcription and translation, this organelle would appear to be autonomous. However, an assessment of the coding capacity of the chloroplast genome suggests that plastid DNA could code for about 125 polypeptides given a molecular weight (MW) of 50 kD (66). This limited coding capacity means that the chloroplast genome cannot be responsible for all of the polypeptides associated with the structure and function of this organelle.

This assessment finds support in the results of earlier genetic experiments, the implications of which have recently been summarized in (110). Breeding experiments using variegated plants indicated that inheritance patterns of aberrant plastids could be uniparental (maternal) or bi-parental. However, neither pattern could be explained by Mendelian rules and so a genetic system other than nuclear became implicated in plastid inheritance. Conversely, other abnormally developed plastids have been studied whereby inheritance of the defect is clearly Mendelian. In barley alone there are at least 200 recessive lethal and conditional chloroplast mutants whose lesions have been traced to nuclear loci (218). The extent of nuclear involvement in chloroplast development raises the question of co-operation and possible co-ordination between these two genetic systems. To address these questions, many methods have been employed to determine the genetic location encoding chloroplast polypeptides.

Chloroplast genome analysis

In organello protein synthesis provides a very direct means of identifying chloroplastic products (63). In this method, isolated chloroplasts are encouraged to synthesize their own products given an energy source such as light and radioactive amino acids. Using this approach, Blair and Ellis (21) and others (27, 85, 212) established the chloroplast as the site of synthesis for the large subunit (LSU) of Rubisco. This system has also been used to identify thylakoid membrane protein(s) migrating with an apparent MW of 32 kD (27, 62, 83, 84), the α, β and ϵ subunits of coupling factor (CF_1) (137), cytochrome b_{559} (237), cytochrome f (58), apoprotein of the P-700-chlorophyll a-protein (CP1) (237,85), both elongation factors EF-G and EF-Tu involved in spinach chloroplast protein synthesis (45), the dicyclohexylcarbodiimide-binding protein (59) identified as subunit III of CF_0 (198), apoproteins for two minor chlorophyll a complexes (86), some ribosomal proteins of 70S ribosomes (67) and evidence that up to ten proteins of the 30S and eight proteins of the 50S ribosomal subunits of spinach 70S chloroplast ribosomes (60) are chloroplast-encoded products.

As useful as *in organello* protein synthesis has become, the failure to detect a polypeptide among the labelled products does not preclude that it may be synthesized by the plastid. It is conceivable that some proteins may only be synthesized during short, discrete intervals of development (84). For example, it has been reported that changes in the synthesis of LSU-Rubisco and a 32 kD thylakoid protein ('Peak D') reflect changes in the levels of their respective translatable mRNAs and that these levels fluctuate depending upon the stage of development of the leaves from which chloroplasts are isolated (184). An additional consideration is that some polypeptides may be synthesized in such minute amounts that they elude detection. Alternatively, some polypeptides may not be synthesized or may be rapidly degraded in the absence of a cytoplasmically-synthesized complement.

Current methods used to decipher the chloroplast genome attempt to overcome these problems and rely upon recombinant DNA technology. These methods have the potential of

providing the most conclusive evidence for identifying chloroplast-encoded products. This approach takes advantage of the relatively compact size of chloroplast DNA (cpDNA). The chloroplast genome is composed of small, closed, circular DNA ranging in size from 80 x 10^6 - 100 x 10^6 D for higher plants (112,200) to the somewhat larger size of *Chlamydomonas reinhardtii* at 126 x 10^6 D (166). For these analyses, cpDNA is digested using various restriction endonucleases. The fragments of DNA are then cloned in bacterial plasmids. The sequence coding for a polypeptide is then identified either by a coupled transcription-translation system or by hybrid-arrest or hybrid-release translation (233). The identity of the polypeptide can be verified by the same techniques used for *in organello* synthesis including immunoprecipitation, peptide mapping and/or migration patterns in polyacrylamide gels.

These methods have been used to locate the gene for the LSU-Rubisco (rbcL) on the chloroplast genome of a number of plants including maize (17,49), spinach (68), mustard (124) and wheat (29). Identification of the 32 kD polypeptide has proven to be somewhat more complex as several chloroplast polypeptides co-migrate on polyacrylamide gels with an apparent MW of 32-34 kD. One of these products, designated 'Peak D' following *in organello* protein synthesis (62), is now more frequently referred to as the "32 kD polypeptide". This product has been identified as the herbicide-binding protein of PSII (155,195). The corresponding gene, psbA has been mapped in several plants (18,61,124) and sequenced (239). Chua and Gillham (42) were able to resolve several proteins in this range including one they named 'D2'. The gene for this polypeptide, psbD, has been mapped and sequenced on the cpDNA of *Chlamydomonas reinhardtii* (167) and pea (160). Examples of other studies which have identified cpDNA genes include locating the site for cytochrome f in pea (234), components of PSI specifically the P700 chlorophyll a apoprotein in spinach (227), pea (189) and maize (74), the α, β and ϵ subunits of CF_1 in spinach (229), subunit III of CF_0 in wheat (97) and pea (101), and chloroplastic EF-G and EF-Tu in spinach (46).

Nuclear genome analysis

A number of experimental approaches have been taken to determine the contribution of cytoplasmic polypeptides towards the complement of proteins found in the chloroplast. The strategies employed have been somewhat analogous to *in organello* studies in that they have been designed to eliminate the contribution of one genome using either intact cells or tissues.

One such approach involves the use of inhibitors which selectively inactivate protein synthesis either by chloroplastic 70S ribosomes or cytoplasmic 80S ribosomes. Despite the concerns which must be addressed regarding the specificity and possible metabolic side-effects of such inhibitors (63,79), studies using inhibitors stongly suggest that the majority of chloroplast polypeptides are synthesized in the cytoplasm (42,63) and are eventually transported to the chloroplast. These results, summarized by Ellis (64) and in (110), provide evidence that most of the Calvin cycle enzymes (with the notable exception of LSU-Rubisco), ferredoxin, RNA polymerase, aminoacyl tRNA synthetases, plastocyanin, ferredoxin-$NADP^+$ oxidoreductase and many ribosomal, thylakoid and envelope polypeptides are synthesized on 80S ribosomes.

Further evidence comes from a physiological approach to studying the same problem. Some higher plants, including many cereals, do not accumulate chlorophyll and are deficient in 70S ribosomes when grown continuously at elevated temperatures (32 to 34°C) (71). The deficiency in 70S ribosomes occurs independent of light conditions. That is, dark-grown heat-treated seedlings are also deficient in 70S ribosomes (71,72), a condition which contrasts with etiolated cereal seedlings which normally possess a considerable complement of plastid ribosomes (71,72).

Feierabend and his co-workers (69,70,71,72,73,) have carried out numerous biochemical and physiological studies in order to characterize these 70S ribosome-deficient plants. In spite of the lack of chlorophyll, the heat-treated seedlings appear to have all of the enzymes necessary for chlorophyll biosynthesis as conversion of exogenously added δ-aminolevulinic acid to protochlorophyllide can be demonstrated (69). Carotenoids are also

present in heat-treated seedlings albeit at relatively lower levels than 22°C control plants (71). A survey of chloroplast enzyme activities from 6 d seedlings grown at 22°C and 32°C suggests that normal levels of NADP-malate dehydrogenase, phosphoglycollate phosphatase, phosphoribulokinase and NADP-glyceraldehyde-phosphate dehydrogenase can be measured but Rubisco activities are barely detectable (71,72).

In some plants, 70S ribosomes fail to accumulate because of a mutation. The *iojap* mutant of *Zea mays* is a nuclear-gene induced plastid genome mutant which phenotypically displays white stripes or completely white leaves. The white sectors do not contain 70S ribosomes (219) nor detectable levels of either Rubisco or CF_1 yet phosphoenolpyruvate (PEP) carboxylase activity was about 40% of that measured using green tissue (182). A somewhat similar *albostrians* mutant of *Hordeum vulgare* is also devoid of plastid ribosomes but exhibits wild-type levels of cytoplasmic ribosomes (25). However, the failure to accumulate 70S ribosomes occurs despite the synthesis of chloroplastic 23S and 16S rRNAs by RNA polymerase (183). Immunological analysis showed that neither Rubisco nor CF_1 are present (25,24).

The ac-20 nuclear mutant of *Chlamydomonas reinhardtii* is also deficient in plastid ribosomes when grown on an organic medium (82). These cells contain little or no Rubisco (80,209) but near normal levels of other Calvin cycle enzymes (209). In contrast, a nuclear gene mutant of barley, *tigrina* o^{34}, exhibits a loss of ribosomes at a restrictive temperature of 20°C (99). When seedlings are grown at the permissive temperature of 30°C, wild-type levels of both ribosomal classes may be found. In contrast to the heat-treated material of Feierabend, developing plastids at the intercalary meristem possess 70S ribosomes but these appear to be lost after assembly at the restrictive temperature. This loss has been attributed to photodestruction as a result of low carotenoid levels (36). As a consequence, profiles of polypeptides analyzed by polyacrylamide gels reflect a marked reduction though not complete loss of a number of polypeptides including CF_1 and several thylakoid membrane proteins (99).

Another approach that has been used to identify nuclear gene products has involved the isolation of messenger RNA (mRNA) and its subsequent *in vitro* translation using a number of

heterologous cell-free protein synthesizing systems. In order to distinguish between cytoplasmic and chloroplastic mRNA, researchers have exploited a feature of plastid RNA that was observed by Wheeler and Hartley (232). They reported that spinach chloroplast RNA does not contain extensive tracts of polyadenylate (poly(A)) residues. The possession of a 3'-poly(A) tail is usually thought to be an eukaryotic trait and may be 20-250 bases long. This distinction is not absolute as a small amount of RNA containing poly(A) tracts has been found associated with chloroplasts of maize (90) and *Euglena* (143). However, the fraction of poly(A) RNA in chloroplasts appears to be minor and does not include the major transcripts such as those for LSU-Rubisco or the 32 kD polypeptide (136,232,239). Consequently, the retention of mRNA species by oligo d(T)-cellulose is generally taken as a strong indication that a particular message is a product of the nuclear genome (140,228). Binding to this matrix usually requires a poly(A) tract of at least 20 bases (188).

As emphasized earlier, the changes that accompany the light-mediated development of a chloroplast can be monitored by the qualitative and quantitative changes in polypeptides. The observation that these changes, particularly qualitative ones, are also reflected among the species of translatable mRNAs was demonstrated in *Lemna gibba* (206) using purified poly(A) RNA translated in a wheat germ cell-free system. Subsequent studies using *in vitro* translation combined with immunoprecipitation identified two very prominent nuclear-encoded products.

Dobberstein *et al.* (57) were able to immunoprecipitate a *Chlamydomonas reinhardtii* poly(A) RNA-directed translation product of MW 20 kD using an antibody directed against the SSU of Rubisco. This polypeptide is larger than SSU by about 3,500 D yet the authors were able to show that this species could be processed to mature SSU size by an unspecified endopeptidase present in a post-ribosomal supernatant of *Chlamydomonas reinhardtii*. These findings were substantiated by other studies showing that a precursor form of SSU (pSSU) could be synthesized using poly(A) RNA from other sources including *Lemna gibba* (203), peas (38,43,95) and spinach (43). The demonstration that incubation of pSSU with intact chloroplasts of pea (43,95) and spinach (43) resulted in uptake and processing to mature SSU

established that synthesis of the higher MW form was not likely an error of translation. These studies also established that transport of pSSU across the membrane was independent of translation.

The second chloroplast-destined polypeptide whose poly(A) RNA *in vitro* translation product suggested significant light enhancement is the apoprotein of the light-harvesting chlorophyll a/b protein (LHCP). As with pSSU of Rubisco, *in vitro* translation of poly(A) RNA using either wheat germ or reticulocyte lysate cell-free systems followed by immunoprecipitation reveals higher MW precursor form(s) of this polypeptide in barley (5,19,149), *Lemna gibba* (205), and pea (52,177). In barley, Apel and Kloppstech (5) reported a single LHCP component of an apparent MW of 25 kD with a precursor of 29 kD while Bellemare *et al.* (19) immunoprecipitated two precursors designated p15 (MW 30 kD) and p16 (MW 29.5 kD) using an antibody prepared against *Chlamydomonas reinhardtii* LHCP. Pea poly(A) RNA-directed products immunoprecipitated by a similar antibody preparation demonstrated that two bands of 32 and 34 kD were synthesized (177). These products could be processed by either pea or lettuce chloroplasts to 15 and 16 kD sizes (177). Also using this antibody, a major 32 kD and minor 31 kD product could be immunoprecipitated in *Lemna gibba* (205). It is interesting to note that these translation products are considered precursors to integral membrane proteins and yet are synthesized as soluble polypeptides (177,178).

In addition to these two rather well-characterized products, *in vitro* translation of poly(A) has provided information about a number of other nuclear-encoded products. Antibodies raised against three chloroplast envelope membrane proteins have been used to identify the 22 kD, 29 kD (phosphate translocator) and 36 kD envelope polypeptides from among those programmed by poly(A) RNA of spinach (75). These polypeptides are synthesized as 32, 40 and 38 kD precursors, respectively. Translation of spinach poly(A) RNA followed by immunoprecipitation has demonstrated that the γ and δ subunits of CF_1 are cytosolic products and are also synthesized as precursors (224).

Synthesis of poly(A) RNA-directed polypeptides followed by chloroplast uptake of the products indicate that as many as 100 soluble and membrane polypeptides may be synthesized in the cytoplasm and transported to the plastid (89). Chloroplast uptake of plastocyanin (10 kD), ferredoxin-$NADP^+$ oxidoreductase (35 kD) and fructose-1,6-bisphosphatase (47 kD) has been reported using pea (89). This study also demonstrates that both plastocyanin and ferredoxin-$NADP^+$ oxidoreductase are synthesized as precursors of about 15 and 8 kD larger than their respective mature proteins. A similar approach showed that spinach poly(A) RNA could be used to direct the *in vitro* synthesis of three polypeptides (24, 23 and 16 kD) which appear to be associated with the water-splitting site of PSII. Precursors 6-10 kD larger than the mature protein were formed for each and successful assembly of the imported proteins was suggested (228). Similarly, a 17 kD chloroplast membrane polypeptide of undetermined function is synthesized by pea poly(A) RNA as a 24 kD precursor (140). For all of these products, transport into the chloroplast is a post-translational event.

Just as light serves to enhance the level of transcripts for pSSU and the apoprotein of LHCP, stimulation by light also increases the level of translatable mRNA for such nuclear-encoded products as NADP malic enzyme (51) and PEP carboxylase (186) in *Zea mays*. Translatable mRNAs for the NADP-dependent chloroplastic glyceraldehyde 3-phosphate dehydrogenase subunits also undergo increases in response to light as indicated by an increase in immunoprecipitable precursors synthesized *in vitro* (39). Interestingly, an analogous response to light is not found for mRNA encoding the cytosolic NAD-dependent species of this enzyme. In addition, the *in vitro* synthesized polypeptides for the cytosolic NAD-dependent dehydrogenase are not synthesized as precursors of higher MW than the mature polypeptide, a feature which was noted for the chloroplast-destined species (39).

In contrast to enhanced transcript levels in response to light, a decrease is found in the level of translatable mRNA encoding NADH-protochlorophyllide oxidoreductase when dark-grown barley is illuminated (3,4,14). This trend is also reflected in the rapid decline in activity and levels of this enzyme following illumination (172). This enzyme catalyzes the

light-dependent reduction of protochlorophyllide to chlorophyllide. Due to the ephemeral appearance of its mRNA, it is suggested that other enzymes are mainly responsible for chlorophyll synthesis and accumulation during later stages of greening (4). As is typical for other chloroplast-destined products, the mRNA codes for a 44 kD polypeptide, about 8 kD larger than the mature protein (3).

C. Regulation of Gene Expression

It is apparent that mRNA plays an important biological role in the transfer and eventual expression of genetic information. As a consequence, isolation and characterization of prevailing mRNA has been used towards understanding the processes which affect gene expression. In plants, the role of light as a stimulus has received much attention because of its instrumental role in greening.

Transcriptional Regulation

Although low amounts of Rubisco may be detected in most dark-grown tissue, illumination brings about an increase in activity which is attributed to *de novo* synthesis of the enzyme (111,157). In *Lemna gibba*, exposure to light leads to enhanced levels of translatable mRNA for pSSU (203,208) as measured by the increase in immunoprecipitable pSSU synthesized by a cell-free translation system. This increase is mediated by phytochrome (204). The development of cloned, complementary DNA (cDNA) probes for pSSU mRNA (16,30,197) has made it possible to use hybridization analysis to measure the steady-state levels of transcripts present. Using this approach, light-stimulated increases of pSSU mRNA were shown using pea (105,192,181,201) and *Lemna gibba* (197). The evidence that these increases reflected enhanced rates of transcription was demonstrated using isolated pea leaf nuclei (78) and *Lemna gibba* nuclei (185). In many of these studies (105,197,185,201), phytochrome involvement has been proposed. In the case of barley (215,217), cucumber (220) and mung bean (201), a less stringent requirement for light in the regulation of pSSU mRNA synthesis is

suggested. Studies using barley (215,217) show that the developmental state of the chloroplast or etioplast may exert a considerable influence over pSSU synthesis. This explanation may also apply to cucumber and mung bean.

Enhanced levels of LHCP mRNA in response to light have been demonstrated with cDNA hybridization analysis using poly(A) RNA of barley (4,81), *Lemna gibba* (197), pea (105,201) and mung bean (201). Regulation at the point of transcription has been shown using pea (78), barley (147) and *Lemna gibba* (185) nuclei. Mediation by phytochrome has been reported for barley, *Lemna gibba* and mung bean (81,147,197,201) but conventional phytochrome involvement has not been demonstrated in pea (105,108,201). In contrast, exposure to light results in a decrease in transcription of the gene for barley NADP-protochlorophyllide oxidoreductase (147) by a phytochrome-mediated mechanism (14).

These results indicate that light can play a direct role in the transcription of cytoplasmic transcripts. Studies on mustard cpDNA using cloned fragments of the chloroplast genome (126) suggest that most regions of this genome are constitutively expressed although the relative abundance of certain transcripts may vary. For example, while substantial levels of rRNA and intermediate levels of LSU-mRNA could be detected in etioplasts, transcript levels for the 32 kD polypeptide were extremely low (126). Transcript levels have been determined for photo-regulated genes in *Zea mays* and increases have been observed for those of the 32 kD polypeptide, LSU of Rubisco, apoprotein(s) of CP1, subunit III of CF_0, in addition to the α, β and ϵ subunits of CF_1 upon illumination. Levels of all of these transcripts except those of the 32 kD polypeptide (psbA gene) showed an eventual reduction upon prolonged illumination (168). While some reports provide evidence for the strict expression of psbA gene by light (18,125,161), transcript levels for this gene and that of the LSU of Rubisco both reached high levels in etiolated mung bean and pea buds (201). Phytochrome involvement for pea (201) and mustard (125) psbA transcript synthesis and rbcL transcript synthesis in pea (174,173) but not mustard (125) have been proposed.

The nature of light induction is currently being addressed using the Ti plasmid of *Agrobacterium tumefaciens* to transform plant cells. Broglie *et al.* (31) have inserted a SSU gene (rbcS-E9) into petunia protoplasts and demonstrated that the inserted gene is transcribed, post-transcriptionally modified, translated and eventually associated with petunia LSU in the chloroplasts of regenerated petunia calli. Of particular interest is the fact that the mRNA of rbcS-E9 becomes regulated by light just as it is in pea. Herrera-Estrella *et al.* (94) constructed a chimaeric gene by linking the promoter sequence of the pea SSU gene to a bacterial gene for chloramphenicol acetyltransferase. Subsequent transformation of tobacco cells using the Ti plasmid imparted light-induced expression of the bacterial gene. This provides compelling evidence that sequences upstream of rbcS are responsible for light induction. Recently (146), the use of 5' deletion mutants of the upstream region of rbcS-E9 suggest that a small, 33 base pair region near the TATA box imparts light regulation.

Post-transcriptional Regulation

Although light has been underlined as a key stimulus for leaf development at the level of transcription (207), regulation of gene expression may be exerted by post-transcriptional means. In eukaryotes, information borne by transcripts often requires post-transcriptional modifications and transport to the cytoplasm where it can be decoded into a specific polypeptide sequence. After translation the polypeptide product may be transported to an organellar destination or otherwise post-translationally modified before a gene is "expressed" in an appropriate physiological role. Regulation in eukaryotes may occur at any, or indeed, all of these steps (32).

One way to assess increases or decreases in the capacity of an organism to synthesize proteins is by analyzing shifts in the proportion and size classes of polyribosomes (polysomes). Polysomes are complexes composed of more than one ribosome which become attached to a strand of mRNA. Generally, longer messages may be associated with many ribosomes giving rise to various size classes designated as "mers" (dimers, trimers, etc). Each ribosome along a

message is involved in translating the polypeptide. Shifts in the relative distribution of these size classes may be analyzed by sucrose density gradient centrifugation. Changes in the absorbance profile of these gradients have been used to assess *in vivo* effects of phytohormones (53,54,40,132,213), heat shock (11,109), water stress (100,134) and other developmental factors such as light (194,211,210) and phytochrome-mediated photomorphogenesis (77,76,148,190).

A number of researchers (55,118,119,148) have raised concerns about the validity of these assessments. They argue that the usual practice of pelleting polysomal preparations through sucrose cushions prior to loading on gradients removes smaller ribosomal particles, especially 80S and 70S monosomes and their subunits. They suggest that these losses lead to a distorted view of the translational capacity of a tissue since this capacity is often reported as a ratio of polysomes to monosomes. These ratios disregard the potential role of subunits, an omission which has also been criticized (148). Further, the contribution of membrane-bound polysomes can be underestimated if detergents are not used in sample preparation (55,118). Artifactual changes in profiles can be minimized by the use of buffers of high pH and high ionic strength (104), chelators such as EGTA which remove both heavy metal ions and can directly inactivate Ca^{++}-regulated ribonucleases (104,117), appropriate Mg^{++} and K^{+} concentrations to maintain stability of ribosomes and any of a number of specific RNase inhibitors such as heparin (159,154) and 2' and 3' mononucleotides (128).

Caution must also be taken in the interpretation of polysome profiles (55). The possession of larger polysomes is not necessarily indicative of increased rates of protein synthesis. Rather, a decreased *in vivo* rate of protein synthesis could be responsible for this shift if the rate of translation initiation was unaltered but the rate of elongation and/or termination decreased. Alternatively, the predominance of longer messages may shift the profile towards larger polysomes without an actual change in the overall rate of protein synthesis. In the heat-shock response of HeLa cells, the shift in polysomes towards larger sizes occurs in spite of a reduced rate of overall protein synthesis. This shift is attributed primarily to the highly

efficient, preferential initiation of heat shock mRNA polypeptide translation (910). Using polysome profile analysis, Mösinger and Schopfer (148) were able to demonstrate that light-mediated increases in both membrane-bound and cytosolic polysomes in mustard is accompanied by increases both in newly synthesized mRNA and the recruitment of ribosomal subunits into polysomes. These changes were demonstrated to be phytochrome induced.

There is increasing evidence that transcriptional control of LHCP mRNA is not the only regulatory point in the accumulation of this polypeptide. In pea, low levels of LHCP transcripts can be readily detected in the dark (52,56,105,201) yet LHCP cannot be found in etiolated seedlings (20). Plants grown under intermittent illumination lack chlorophyll b (7) and LHCP fails to accumulate in thylakoid membranes despite the fact that mRNA for this polypeptide is associated with polysomes (20,52). Selective inhibition of the translation of these messages could not be demonstrated (52). Analysis of cell-free translation products of a chlorophyll-b less *chlorina f2* mutant of barley showed that translatable mRNA was present (5,19). This mutant lacks both chlorophyll b and LHCP (221) and yet products synthesized by LHCP mRNA could be transported and incorporated into wild-type or mutant chloroplast membranes (19). Similarly, LHCP mRNA is associated with polysomes and is apparently translated in a chlorotic pea mutant and a chlorophyll-b less pea mutant (180). However, LHCP accumulates in the thylakoid membranes at a much reduced rate or imperceptibly in the case of the chlorophyll-b-less mutant. It has been proposed that after transcription and translation, the apoproteins of LHCP polypeptides are transported into the chloroplast and inserted into thylakoid membranes (178) but are rapidly degraded if both chlorophylls a and b are not available to post-translationally stabilize the product (5,20,52). Differential accumulation of several chlorophyll a/b binding polypeptides in the *chlorina f2* barley mutant suggest different roles for chlorophylls a and b (19). Furthermore, the presence of LHCP mRNA in chlorophyll-deficient maize yet its absence in carotenoid-deficient maize led authors to suggest that the extent of plastid development may affect transcription of LHCP mRNA (135). The plastids of carotenoid-deficient seedlings are devoid of internal structures.

Controls may also exist by regulating the transport of a newly-synthesized polypeptide to its final destination. Transport may involve transit across one or more membranes followed by insertion into a membrane as is required for the water-splitting site polypeptides (228).

Mitochondrial and chloroplast-destined (44,175) proteins appear to be synthesized as higher MW precursors. These precursors are able to bind to the outer membrane such as the chloroplast envelope (47,156) and transit across the membrane occurs by an energy dependent step (47,88). Polypeptide uptake studies support transport by post-translational mechanisms as opposed to the co-translational transport process found for nascent secretory polypeptides synthesized by membrane-bound ribosomes on rough endoplasmic reticulum (22,170).

Precursors are bound to the chloroplast envelope in preference to the mature polypeptide (156) and the existence of receptor sites composed of protein has been proposed (47). The extra sequence or "transit sequence" of pSSU bears a positive charge (102) and this may play a role in import. Comparison of the transit sequences for pSSU and the apoprotein of LHCP does not reveal extensive homology although both appear to be rich in serine residues near the NH_2 terminus (37,145). The poor homology may reflect different processing required by soluble and membrane-bound polypeptides. Alternatively, there is some evidence that binding receptors for the transport of proteins into mitochondria can be very specific (175,238). Yaffe and Schatz (236) reported on two conditional, temperature-sensitive yeast mitochondrial protein transport mutants. These mutations are pleiotropic in that the defective transport mechanism inhibits the import of several precursors but transport of different precursors appear to be inhibited in the two strains.

Smith and Ellis (191) suggest that the processing of pSSU and assembly of the holoenzyme takes place in the chloroplast stroma. A partial purification of a soluble, chloroplastic metal-ion-dependent protease prepared from peas has been reported (164). This enzyme was capable of processing pea pSSU and polypeptide precursors of wheat and barley including pre-plastocyanin and pre-ferredoxin-$NADP^+$ oxidoreductase. The processing of pSSU may occur as a two step process (165). The actual proteolytic processing is probably not the

energy-dependent step of precursor uptake (164).

D. Control and Co-ordination of Protein Synthesis

It is clear that genes for the soluble and insoluble polypeptides engaged in activities as diverse as CO_2 fixation and mRNA translation are essentially dispersed between the nuclear and chloroplast genomic systems. An additional degree of complexity is evident in that many of the nuclear coded products form functional units only after being united with a chloroplast-encoded product. A well-studied example of this arrangement is the enzyme Rubisco. It is tempting to speculate that controls must exist to co-ordinate the activities of these two centers yet our understanding of this regulation is still very poor.

Rubisco has served well as a model of nuclear-chloroplast interactions due, in part, to its abundance and partly because its subunits are conveniently synthesized in two separate compartments. However, evidence for co-ordination between the synthesis of LSU and pSSU has remained somewhat contradictory. Although tight-coupling has been proposed for *Chlamydomonas reinhardtii* (103) and *Chlorogonium elongantum* (231) using inhibitors, other studies which demonstrate the synthesis of LSU in the absence of pSSU synthesis in soybean cells (13) or the apparent accumulation of pSSU in pea leaves (169) and chloroplast ribosome-deficient rye (73) suggests that co-ordination may not be strict. A study using the *Chlamydomonas reinhardtii* ac 20 mutant grown under conditions that eliminate or inhibit 70S ribosomes suggests that no SSU can be detected (80). More recently, Mishkind and Schmidt (144) reported that wild-type levels of pSSU mRNA could be found in this mutant and that transcripts are associated with polysomes and ribonucleoproteins. The failure to detect this product is attributed to rapid degradation of unassembled pSSU in the chloroplast (179).

There is some evidence that increased synthesis of pSSU mRNA in light is paralleled by increases in LSU mRNA for pea (105,181,174,173,192) and *Zea mays* (168). The exact nature of control mechanisms has not been determined.

III. Materials and Methods

A. Plant Material

Seeds of *Hordeum vulgare* L. cv. Gateway (N) and its *virescens* mutant (M) were surface-sterlized by soaking for 10 min in 5%(v/v) NaOCl followed by extensive rinsing with distilled water. The seeds were then sown in trays containing vermiculite and watered daily with tap water. For light-grown seedlings, trays were transferred to a growth cabinet providing continuous illumination at a PAR intensity of 100 μE $m^{-2}s^{-1}$ and a constant temperature of 20±1°C. Seedlings were harvested 4, 5 or 6 d after sowing. Etiolated material was obtained by germinating the seeds in a dark cabinet for 4 d at 20±1°C. These seedlings were harvested in a dark room under a very dim, green safelight.

Routinely, seedlings were harvested immediately before being used. However, in the case of plant material used in the generation of polysome profiles, the harvested tissue was weighed, separated into 1.0 g packages, rapidly frozen in liquid N_2 then stored at -80°C. It was found that material stored at this temperature gave reproducible polysome profiles providing the material was not subjected to a freeze-thaw cycle.

The barley leaf, like leaves of other monocotyledons, grows from a basal meristem so that the growing leaf represents a gradient of development with respect to plastids, pigments, photochemical actvity (10,122,163) and gene expression (215). Consequently, only about 1.0-1.5 cm apical leaf segments were harvested in order to minimize developmental variation within the plant material sampled.

All efforts were made to ensure the uniformity of environmental conditions between harvest dates as earlier studies have shown the mutant to be both light and temperature sensitive (142). Towards this end, chlorophyll a and b measurements (8) and fresh and dry weight determinations were taken to help evaluate the maturity and growth of the seedlings. Also, all studies comparing the N and M were carried out in parallel.

B. Materials

Precautions were taken to minimize RNA degradation in an effort to maximize yield and translatability of the isolated product. All reagents used were either of the highest quality commercially available or were purified, redistilled or recrystallized before being used. Water used alone or to make solutions and buffers was deionized and double-distilled. Also, the water, solutions and buffers were sterilized by autoclaving or, more generally, by filtration through a 0.22 μm filter. The glassware, mortars and pestles were sterilized by autoclaving and then baked at 200°C for several hours to further reduce the chance of RNase contamination. Plasticware and tubing were autoclaved when possible or, alternatively, heated in 1%(w/v) SDS at 60°C for at least 15 min then rinsed extensively with water. The inadvertent introduction of pancreatic RNase which is found in perspiration (91) was prevented by wearing disposable gloves at all times.

C. Isolation of RNA

Extraction of Total RNA

Following harvest, apical laminae segments were rinsed with water then blotted dry using paper towelling. Routinely, 10 g of this tissue was ground to a fine powder in liquid N_2 using a mortar and pestle. The powder was then transferred to a flask, suspended in 5 vol of extraction buffer containing 50 mM Tris·HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 40 μg/ml proteinase K (Sigma, Type XI) and 2%(w/v) SDS (57) then shaken at room temperature on a rotary shaker. After 30 min, 0.5 vol of buffered phenol containing 0.1%(w/w) hydroxyquinoline (129) was added. This mixture was shaken vigourously and allowed to sit for 2-3 min before a similar volume of chloroform:isoamyl alcohol (49:1, v/v) was added and shaking continued for 30 min.

Separation of the phases was completed by a low speed centrifugation at 1000*g* for 5 min at 3-4°C. The upper, aqueous phase was withdrawn and set aside while the lower, organic

phase was re-extracted with a further 0.5 vol of extraction buffer. Following phase separation, the aqueous phases were pooled and extracted with an equal volume of buffered phenol:chloroform:isoamyl alcohol (50:49:1, v/v/v) until the flocculent interface of denatured protein and cellular debris was no longer apparent. This generally required two additional extractions. The aqueous phase was then extracted with an equal volume of chloroform:isoamyl alcohol (49:1, v/v) to remove as much of the phenol as possible. At this point, 2.5 vol of ethanol were added to the aqueous phase and the nucleic acids precipitated at -20°C overnight.

The precipitate was pelleted by centrifugation at 10,000*g* for 10 min at 0°C. The pellet was washed twice using a buffer of 3 M sodium acetate (pH 6.0) and 10 mM EDTA (230). Washes were carried out by gently suspending the pellet in a small volume (usually 5 ml) of chilled buffer and letting the mixture sit on ice for 15-20 min. The nucleic acids were then pelleted by centrifugation at 23,000*g* for 15 min at 0°C. These washes serve to remove much of the DNA, tRNA, some rRNA and contaminating polysaccharides (199). A third and final wash using 70%(v/v) ethanol was completed before the pellet was dried by a stream of N_2(g). The dried pellet was dissolved in a minimal volume of 0.3 M NaCl and the RNA precipitated with 2.5 vol of ethanol overnight.

The precipitated RNA was collected by centrifugation at 10,000*g* for 10 min at 0°C. The pellet was washed once with 70%(v/v) ethanol and dried with N_2(g). The dried pellet was then dissolved in water (usually 5 ml) and prepared for fractionation into poly(A) and non-poly(A) containing RNA.

Poly(A) RNA Separation

RNA bearing polyadenylate tracts or poly(A) RNA was separated from non-polyadenylated RNA by affinity chromatography using oligo(dT)-cellulose (Sigma) (9). The procedure employed was a modified version of the one outlined by Maniatis *et al.* (129). All steps were carried out at room temperature.

The sample of RNA dissolved in water was heated for 5 min at 65°C at the end of which an equal volume of 2X Column Loading Buffer was added. Column Loading Buffer was composed of 20 mM Tris·HCl (pH 7.6), 0.5 M LiCl, 1 mM EDTA and 0.1%(w/v) SDS. This solution was allowed to cool before being applied to a column of oligo(dT)-cellulose equilibrated with Column Loading Buffer. Once applied, the sample was slowly circulated through the column for 15 min to encourage maximal binding of poly(A) RNA. To facilitate collection of nucleic acids, the eluate was monitored at 254 nm using a Gilson Model III UV monitor. Unbound nucleic acids were collected and the column washed until no further UV-absorbing material was observed in the eluate. The poly(A) RNA was then eluted from the column using a 10 mM Tris·HCl (pH 7.5), 1 mM EDTA and 0.5%(w/v) SDS buffer. The pooled poly(A) and non-poly(A) RNA fractions were adjusted to 0.3 M sodium acetate using 3.0 M sodium acetate (pH 6.0) and 2.5 vol of ethanol added to precipitate the RNA overnight at -20°C. The poly(A) RNA was further purified by repeating the affinity chromatography step on oligo(dT)-cellulose. The second cycle led to a substantial reduction in contaminating rRNA.

Following chromatography, precipitates of poly(A) and non-poly(A) RNA were recovered by centrifugation at 10,000*g* for 20 min at 0°C. The pellets were washed with 70%(v/v) ethanol, dried with N_2(g), then dissolved in a small volume of water. Aliquots were removed for quantification and purity determinations.

Quantification, Purity and Storage of RNA

The yield and purity of all preparations were determined by absorbance readings taken at 260 and 280 nm. An OD reading of 1.0 at 260 nm was taken to correspond to an RNA concentration of 40 μg/ml (129). The ratio of the absorbance readings at 260 and 280 nm (OD 260/OD 280) provided an estimate of the quality of the preparation as a pure solution of RNA should give a value of two (129,188). Poly(A) RNA samples that did not achieve a ratio of 1.8 or better were discarded.

All RNA preparations were divided into small aliquots and stored at -80°C. Samples were only thawed once for use in *in vitro* translation in order to mimimize degradation which inevitably occurs during freeze-thaw cycles.

D. Cell-Free Protein Synthesis

Wheat Germ Cell-Free System

Preparation of Lysate

Raw wheat germ used in the preparation of a cell-free protein synthesizing system was supplied by Mr. Stephens, Executive Director, International Grains Institute, Winnipeg, Manitoba.

The procedure used to prepare the wheat germ lysate was essentially that described by Marcu and Dudock (131). However, the column elution buffer was modified to favour acetate as the counter ion since Cl^- tends to inhibit protein synthesis (225) and the DTT content was doubled to 2 mM. In a typical extraction, 2.0 g of wheat germ were ground with 2.0 g of powdered glass on ice using a mortar and pestle. After 1 min, 4 ml of chilled extraction buffer (20 mM Hepes·KOH, pH 7.6; 100 mM KCl; 2 mM $CaCl_2$; 1 mM magnesium acetate and 1 mM DTT) were added and the resulting paste centrifuged at 24,000*g* for 15 min at 4°C. The supernatant was carefully withdrawn avoiding both the pellet and a viscous, floating lipid layer. One ml of this extract was passed through a 21 X 1 cm Sephadex G25(medium) column equilibrated with 20 mM Hepes·KOH (pH 7.6), 120 mM potassium acetate, 5 mM magnesium acetate and 2 mM DTT. The extract was eluted at a rate of 3.0 ml·min^{-1}. Following elution, the tubes containing the most turbid fractions were pooled usually yielding about 3 ml of extract. This pooled extract was then centrifuged at 24,000*g* for 20 min at 4°C. The supernatant was used for absorbance measurements and the remainder stored as aliquots at -80°C. Aliquots of lysate were thawed and used only once for assays. Each lysate preparation was assessed for

translational activity. Preparations with high endogenous activity or those which did not respond well to exogenous mRNA were discarded.

Protein Synthesis In vitro

Assays were generally carried out using a final volume of 25 μl. Each assay contained 20 mM Hepes·KOH (pH 7.5), 1 mM ATP (diNa salt,Sigma), 0.12 mM GTP (Li salt, Sigma), 8.4 mM creatine phosphate (Sigma), 40 μg/ml creatine phosphokinase (Sigma), 2 mM DTT, 2 mM magnesium acetate, 130 mM potassium acetate, 10 μg/ml human placental RNase inhibitor (Sigma) (176), 24 μM each of 19 unlabelled amino acids (excluding the radio-labelled amino acid), 0.3 vol wheat germ lysate and 5 μl of either L-[^{35}S] methionine (>800 Ci/mmol) or L-[3,4,5-^{3}H(N)] leucine (>110 Ci/mmol) (New England Nuclear Canada, Ltd.). Optimal Mg^{++} and K^{+} concentrations were based upon maximal incorporation of radioactivity into TCA-insoluble polypeptides.

The *in vitro* translation assays were initiated by the addition of water (control) or RNA and allowed to incubate for 60 min at 25°C. Assays were terminated by placing the reaction tubes on ice.

Rabbit Reticulocyte Lysate Cell-Free System

A commercial preparation of reticulocyte lysate was purchased from New England Nuclear. The assays were conducted using L-[^{35}S] methionine (>800 Ci/mmol) and followed the protocol recommended by the manufacturer. The optimal concentrations for potassium acetate and magnesium acetate were determined to be 70 mM and 1.5 mM, respectively. *In vitro* translation assays were incubated at 37°C and terminated by placing the reaction tubes on ice.

Incorporation Measurements

In order to estimate the incorporation of radioactivity into TCA-insoluble polypeptides, duplicate aliquots of the reaction mixtures were removed and spotted onto GF/A

glass fibre filters (Whatman). After the filters were air dried they were dropped into 10%(w/v) TCA containing 0.1 M unlabelled leucine or methionine. The filters were then washed using the method described by Mans and Novelli (130). However, in order to improve the counting geometry of what is basically a heterogenous counting system, the ether-dried discs were processed further following a procedure recommended by New England Nuclear. The filters were transferred to glass liquid scintillation vials, 0.5 ml of Protosol (New England Nuclear) was added and the vial incubated at 55°C for 30 min. The samples were cooled, neutralized with 20 μl of glacial acetic acid, shaken with 10 ml of Econofluor (New England Nuclear), then counted in a Searle Isocap 300 Liquid Scintillation Counter.

Analysis of Products

Electrophoretic Separation of Products

Radioactive polypeptides were analyzed on 7.5-15% SDS-PAGE linear gradient slab gels (41) using the discontinuous buffer system of Neville (152). Electrophoresis was carried out using the Protean 32 cm Model cell (Bio-Rad Laboratories, Richmond,CA) powered with a Buchler 3-1500 Constant Power Supply. The SDS-PAGE molecular weight standards included [methyl-^{14}C]methylated β-lactoglobulin A (MW 18,367), [methyl-^{14}C]methylated carbonic anhydrase (MW 30,000), [methyl-^{14}C]methylated ovalbumin (MW 46,000), [methyl-^{14}C]methylated albumin (bovine serum) (MW 69,000) and [methyl-^{14}C]methylated phosphorylase B (MW 97,400) all from New England Nuclear.

The resolving gel (0.15 X 10.0 X 27 cm) was buffered with 0.4244 M Tris·0.0308 M HCl (pH 9.18) and the 6% acrylamide stacking gel was buffered with 0.0541 M Tris·0.0267 M H_2SO_4 (pH 6.1). The upper reservoir buffer was 0.041 M Tris·0.04 M boric acid (pH 8.64), 0.1% (w/v) SDS while that of the lower reservoir was the same as the resolving gel buffer.

Samples were prepared for electrophoresis by removing aliquots from the assay vials and mixing them with 3 to 5 vol of a sample solubilizing buffer (50 mM Tris·HCl, pH 7.5;

1%(w/v) DTT or 1%(v/v) β-mercaptoethanol; 10%(v/v) glycerol; 2%(w/v) SDS and 0.0005%(w/v) bromophenol blue). The samples were then heated at 90°C for 3 min. After loading the samples, electrophoresis was carried out at 32 ma for 9.5 to 10 h.

After electrophoresis the gels were stained overnight in 0.1%(w/v) Coomassie Brilliant Blue R250 (Sigma), 25%(v/v) isopropanol, 10%(v/v) acetic acid and 0.1%(w/v) cupric acetate and subsequently destained with 40%(v/v) methanol , 7%(v/v) acetic acid. These procedures were required to remove unincorporated amino acids from the gels. The gels were soaked in destain solution containing 3%(v/v) glycerol for at least 1 h followed by ENlightening (New England Nuclear) for 15 min. The first procedure prevents the gel from cracking during drying (223) while the second enables detection of labelled polypeptides by fluorography. The gels were dried down between sheets of cellophane (223) and exposed to Kodak XAR X-ray film at -80°C for varying lengths of time (23). The X-ray film was developed using standard procedures outlined by the manufacturer.

Immunoprecipitation of In vitro *Translation Products*

Antiserum against Rubisco from rye was a generous gift of Dr. J. D. Bewley, Dept. of Biology, University of Calgary. The non-immune serum, 10% suspension of formalin-fixed *Staphylococcus aureus* cells (Cowan 1 strain) and the protocol for immunoprecipitation were provided by Mr. B. Grumbles, Dept. of Genetics, University of Alberta.

Aliquots of *in vitro* translation assays were diluted with an equal volume of 40 mM Tris·HCl (pH7.4), 2.5 mM EDTA, 200 mM NaCl, 0.2%(v/v) Nonidet P-40 (Sigma). This mixture was then incubated with 5 μl of non-immune serum for 15 min at 4°C. The antibody-antigen complex was precipitated using 50 μl of the *Staphylococcus aureus* cell suspension and a 60 min incubation at 4°C and the precipitate pelleted by a 5 min centrifugation at 12,000*g*. The supernatant was withdrawn and incubated for 30 min at 20°C with 10 μl of anti-Rubisco serum followed by an additional 60 min incubation at 4°C. The antigen-antibody complex was precipitated using 100 μl of the *Staphylococcus aureus* preparation followed by

incubation at 4°C for 20 min. The precipitate was collected by centrifugation at 4°C using an Eppendorf microfuge. The pellet was washed once in TEN buffer composed of 20 mM Tris·HCl (pH7.4), 1.0 mM EDTA, 100 mM NaCl and 0.1%(v/v) Nonidet P-40; twice using a buffer of 40 mM Tris·HCl (pH7.4), 1.0 mM EDTA, 1.0 mM KCl and 1.2%(v/v) Nonidet P-40; once in TEN buffer without Nonidet P-40 and finally, once in a buffer of 20 mM Tris, 1.0 mM EDTA and 10 mM NaCl. All washes were done on ice. After the final wash, 50 μl of SDS-PAGE sample solubilizing buffer was added and the sample heated at 90°C for 5 min to dissociate the antigen-antibody precipitate from the *Staphylococcus aureus* cells. The cellular debris was removed by a rapid 30 s centrifugation using an Eppendorf microfuge. The supernatant was then quickly loaded onto SDS-PAGE 7.5-15% polyacrylamide gradient gels and electrophoresis carried out as described earlier. Immunoprecipitates were detected by fluorography of dried gels using varying periods of exposure.

E. Polyribosome Analysis

Polysome Extraction

Polysomes were extracted by rapidly grinding 1.0 g of frozen leaf segments in liquid N_2 using a mortar and pestle until a fine powder was produced. The powder was transferred to a centrifuge tube and extracted with 5 ml of buffer containing 200 mM Tris·acetate (pH 8.5), 200 mM sucrose, 25 mM magnesium acetate, 60 mM KCl, 5 mM DTT, 5 mM EGTA, 2 mM AMP (2′ and 3′ mixed isomers, Sigma) and 0.5%(v/v) Nonidet P40 (Sigma) for 15 to 20 min on ice. The brei was cleared by centrifugation at 24,000*g* for 15 min at 4°C. As this pellet was quite loose, the supernatant was centrifuged an additional 10 min at 32,000*g*. The supernatant was carefully withdrawn and immediately used for polysome profile gradients.

Polysome Gradients

The sucrose gradients used in this study were a slightly modified version of those described by Mösinger and Schopfer (148). For analysis, 10 to 60%(w/w) sucrose gradients buffered with 40 mM Tris·HCl (pH 8.5), 10 mM $MgCl_2$, 20 mM KCl and 1 mM EGTA were made using an ISCO Model 570 gradient former. The 11 ml gradient was layered above a 0.5 ml 65%(w/w) buffered sucrose cushion in 13-ml capacity nitrocellulose tubes (Beckman). The gradients were chilled to 4°C before being overlayed with 1.0 ml of polysomal extract. The gradients were then centrifuged at 25,000 rpm at 4°C for 9.5 h using an SW 27.1 rotor (Beckman).

Following centrifugation, the nitrocellulose tubes were pierced and the gradients eluted from the bottom with the aid of a peristaltic pump. The eluate was monitored continuously at 254 nm using a Gilson Model III UV monitor and the absorbance profile recorded. Fractions were collected and stored at -80°C for subsequent RNA extraction and *in vitro* translation. Polysome gradients were repeated at least three times for all samples. Gradients overlayed by the polysome extraction buffer were used in each experiment to obtain a baseline absorbance profile.

Polysomal RNA Extraction

An equal volume of 50 mM Tris·HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 40 μg/ml proteinase K and 2%(w/v) SDS was added to each gradient fraction. The next steps in the procedure were as already described for Total RNA isolation. However, after the final chloroform:isoamyl alcohol (49:1, v/v) extraction, 0.1 vol of 3 M sodium acetate (pH 6.0) and 10 μg of carrier tRNA (wheat germ deacylated tRNA, Sigma) were added to the aqueous phase before the nucleic acids were precipitated at -20°C with 2.5 vol of ethanol. After pelleting the precipitate by centrifugation at 10,000*g* for 10 min at 0°C, the pellet was washed once with 70%(v/v) ethanol and dried. The dry pellet was solubilized in water, made 0.3 M with respect to sodium acetate (pH 6.0) and precipitation carried out as before. This step was necessary to

remove all traces of phenol and SDS. Finally, the nucleic acids were pelleted, washed with 70%(v/v) ethanol, dried, then solubilized in 10 μl of water. As very little volume was obtained, no measurements were taken of the fractions. Rather, an arbitrary 3 μl aliquot was generally used in *in vitro* translation assays.

F. Total Ribonuclease Activity

RNase activity was measured in crude, soluble extracts obtained from both N and M seedlings grown for either 4 or 6 d under continuous illumination. The assay used to determine RNase activity was the method outlined by Altman *et al.* (2).

For these measurements, 3.0 g of tissue were ground in a mortar and pestle using 7.0 ml of cold grinding buffer (0.002 M sodium phosphate, pH 7.6; 0.1 M NaCl) and a small quantity of acid washed sand to help homogenize the tissue. The brei was centrifuged at 2,300*g* for 15 min at 4°C to remove large debris. The supernatant was then centrifuged at 13,200*g* for 20 min at 4°C. A 2.5 ml aliquot of this supernatant was passed through a PD-10 column (Pharmacia) and eluted with 3.5 ml of grinding buffer. All procedures were carried out at 4°C.

Each RNase assay consisted of 100 μl of diluted crude extract and 400 μl of RNA substrate. The substrate contained 15 mg of purified yeast RNA (Worthington Biochemical Corporation) dissolved in 16.0 ml of 62.5 mM sodium acetate, pH 5.5 buffer. The assays were incubated at 37°C for 30 min and terminated by adding 0.5 ml of 2.5%(w/v) TCA, 0.3%(w/v) lanthanum nitrate to precipitate undegraded RNA. Each assay was done in duplicate and tubes to which the termination solution was added before the extract served as controls. Assay tubes were stored at 4°C overnight then centrifuged for 2 min at 10,000*g* using an Eppendorf microfuge. The supernatant was used for absorbance measurements at 260 nm.

IV. Results and Discussion

A. Characterization of Plant Material

As emphasized in the "Materials and Methods", all of the analyses conducted for these studies were restricted to the apical segments of barley laminae. Consequently, the general growth characteristics of the apical leaf segments were evaluated with respect to chlorophyll and dry matter accumulation.

When seedlings were grown in the dark, N and M seedlings bore a strong resemblance with respect to seedling height and general appearance. This similarity was evident in the equal extent of dry matter (Fig. 1) observed for these seedlings after 4 d growth in continuous darkness. However, although N and M seedlings grown under continuous illumination exhibited comparable leaf expansion and vigour, a significant difference in dry weight between the two seedlings is apparent as early as 4 d. Furthermore, as has been well documented by previous studies (142,127), M seedlings exhibited a marked temporal lag in the accumulation of both chlorophylls a and b as shown in Figure 2. Under the conditions of temperature, light intensity and light quality used for this study, a recovery of approximately 60% of the N total chlorophyll levels was observed in the M by 7 d of development (Fig. 3). In contrast, Figure 2 demonstrates that levels of both chlorophylls a and b were barely detectable at 4 d.

Another feature which distinguished the M from the N was the very high ratio of chl a : chl b observed for the M at 4 d (Fig. 4.) This ratio rapidly dropped by 5 d of development and by 6 d the chl a/b ratio stabilized at a value of about 3.0. These changes contrasted with the rather constant chl a/b ratio of normal seedlings which generally remained around 3.2 for all of the seedling ages studied. The high M ratio of chl a : chl b at 4 d indicates that the rate of chl a accumulation was initially higher than that of chl b in M seedlings. High ratios of chl a :chl b have also been observed during the early phases of greening of etiolated plants (93), etiolated plants grown under intermittent illumination (7) and some mutants as demonstrated by work completed in this laboratory comparing early greening stages of N and M

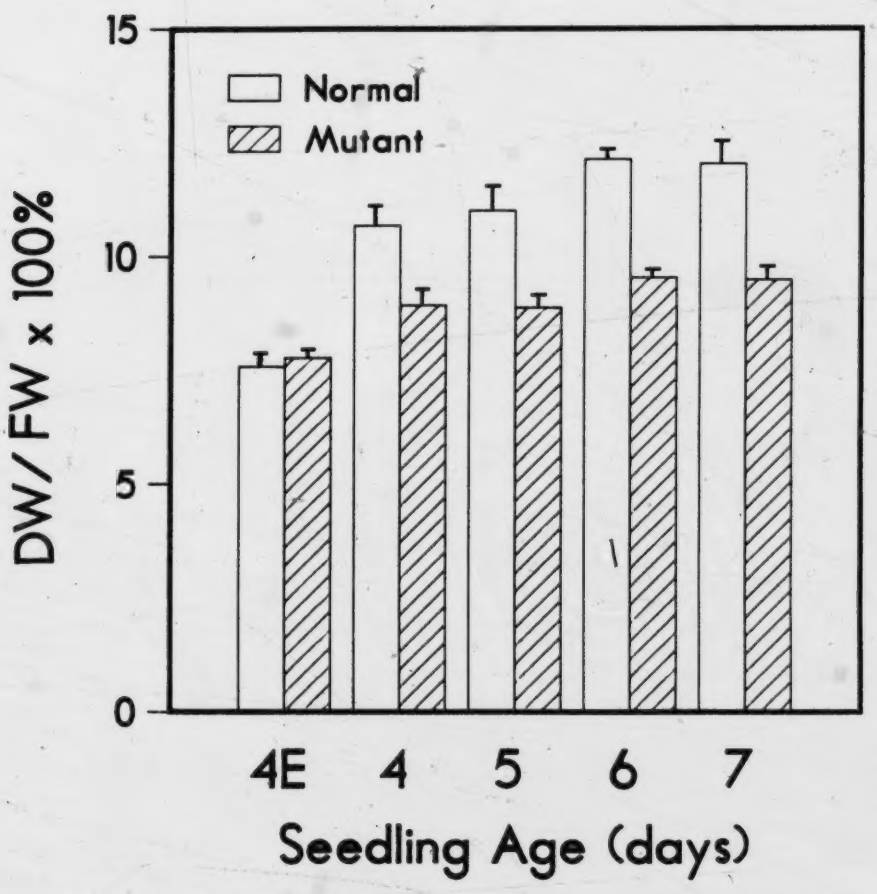


Figure 1. Dry weight determinations of apical leaf segments expressed as a % of fresh weight measurements. Data is based on at least 6 determinations. Bars represent +2 SE. 'E' designates etiolated seedlings.

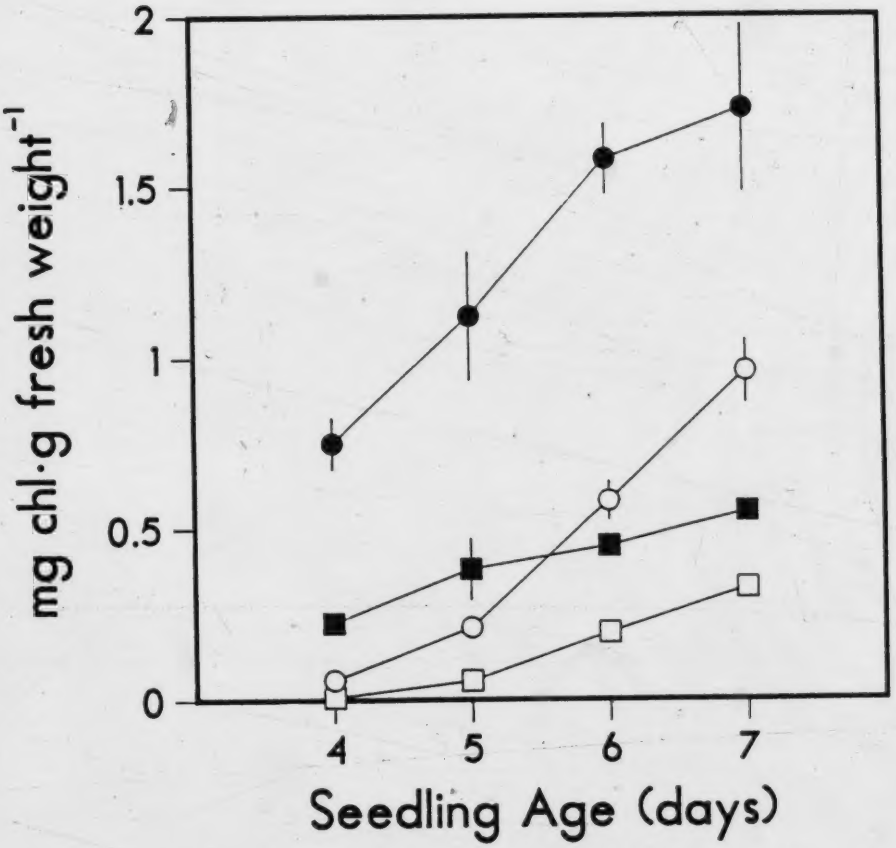


Figure 2. Chl a and b accumulation in apical laminae segments of N and M barley seedlings grown under continuous illumination. N chl a (●), N chl b (■), M chl a (○), M chl b (□). Data is the mean value of at least five determinations. Bars represent ± 2 SE.

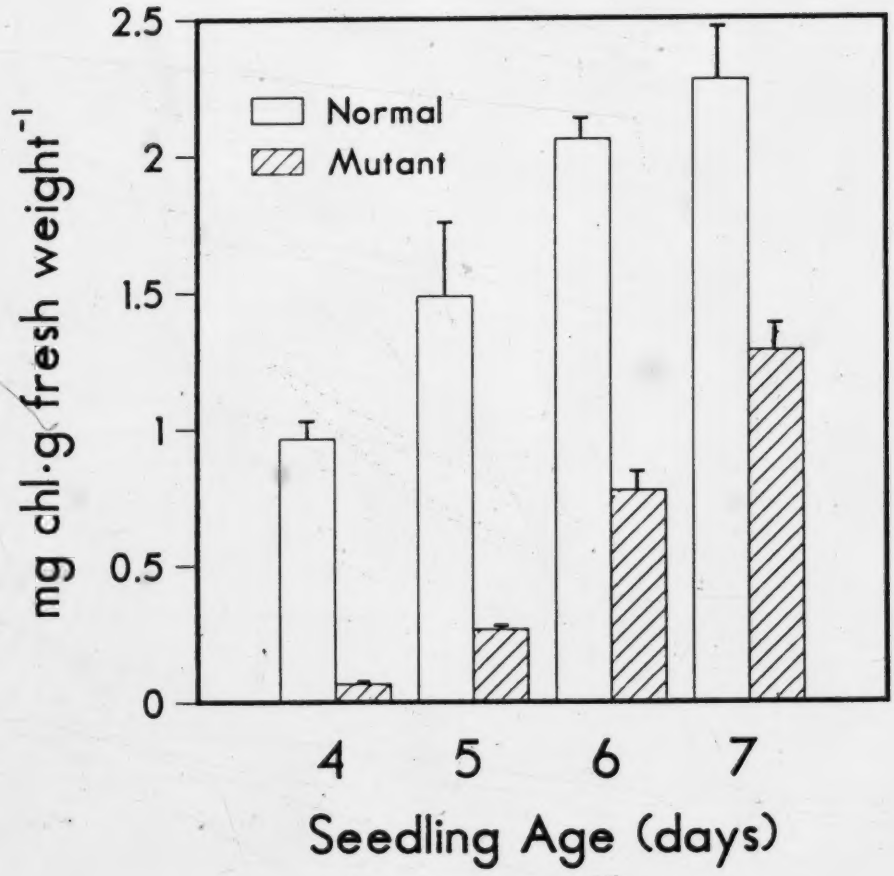


Figure 3. Total chl accumulation in apical laminae segments of N and M barley seedlings grown under continuous illumination. Chl content based upon at least five determinations. Bars represent +2 SE.

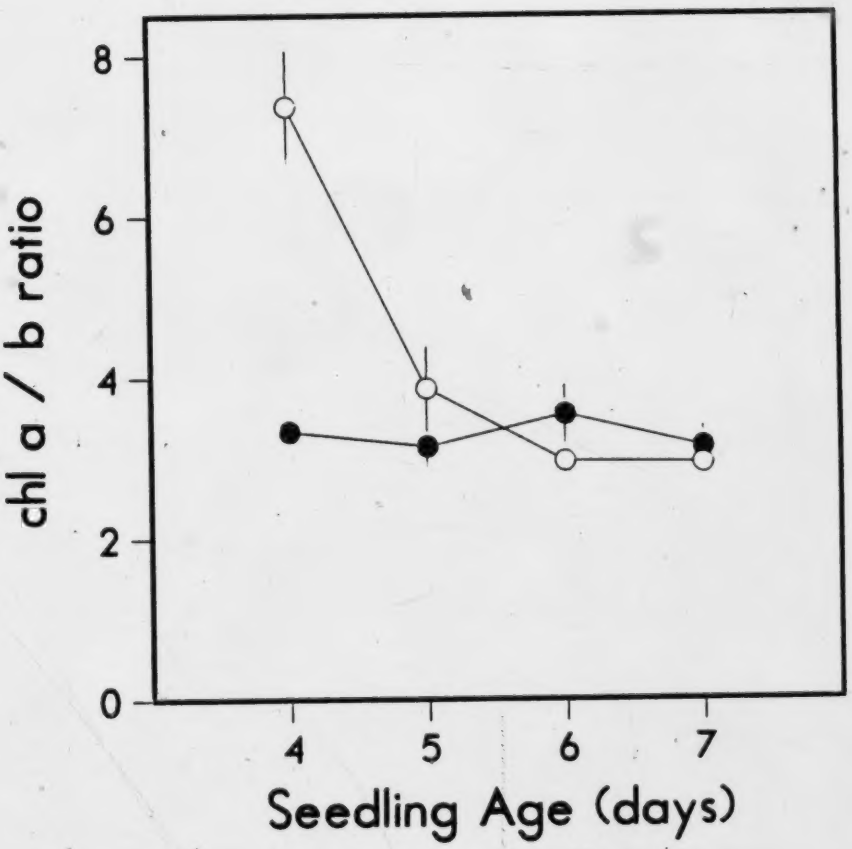


Figure 4. The ratio of chl a to chl b in apical laminae segments of N (●) and M (○) barley seedlings grown under continuous illumination. Data represents the mean value of at least five determinations. Bars represent ± 2 SE.

seedlings (116) in addition to work carried out using other *virescens* mutants (1). However, a high chl a/b ratio is not an inevitable feature of *virescens* mutants as a recent report describes a *virescens* mutant of *Zea mays* which displays a low chl a/b ratio even in partially greened leaf tissue (158).

B. Poly(A) RNA Analysis

Poly(A) RNA Yields

A study completed earlier in this laboratory (141) provided evidence that M seedlings possess less RNA than do those of the wild-type. The RNA content of the M was reported to be approximately 75% that of N seedlings. Furthermore, some anomalous properties in the migration patterns of polypeptides extracted from N and M cytoplasmic ribosomes (114) suggested that the capacity to synthesize proteins might vary between the two seedlings. As ribosome assembly into polysomes might therefore be affected, the method adopted for isolating and purifying poly(A) RNA from the two sources involved total RNA isolation as opposed to the isolation of poly(A) RNA from a polysomal preparation. This strategy was adopted in order to maximize the yield of RNA and to provide the advantage of isolating poly(A) RNA from both N and M which might be translatable but not associated with ribosomes.

Initial attempts to isolate RNA from barley using buffers without proteinase K (28) and the method of Palmiter (154) using heparin as an RNase inhibitor, proved unsuccessful. RNA yields were typically low and subsequent poly(A) RNA preparations were not very active in directing protein synthesis in a wheat germ cell-free system. Rather, use of the buffer system described by Dobberstein *et al.* (57) which employs a combination of SDS and proteinase K for inactivating RNase and deproteinizing the very fibrous homogenate of barley tissue, gave consistently higher yields of total RNA from which poly(A) RNA could be easily purified.

Although no rigorous efforts were made to quantify the amount of poly(A) RNA from N and M seedlings, a consistent observation made was that yields of poly(A) RNA isolated and purified from the M proved to be equal to or often better than those achieved from N leaf segments (Table 1). As discussed in the preceeding paragraph, the inclusion of 2%(w/v) SDS and a rather active, non-specific protease (proteinase K) (188) gave a product with an A_{260}/A_{280} which approximates that of a highly pure solution of RNA ($A_{260}/A_{280}=2$) suggesting that deproteinization of ribonucleoproteins from both samples has met with equal success.

A possibility that was addressed was that N seedlings may have higher RNase activity and that incomplete suppression of these enzymes during initial stages of RNA purification might lead to reduced yields of N poly(A) RNA. This would be in keeping with an early study (120) which reported that a high RNA content is paralleled by high RNase activity in growing barley coleoptiles. However, two observations suggest that incomplete RNase suppression did not compromise N RNA yields. Table II provides estimates of total RNase activity in crude extracts of 4 and 6 d N and M seedlings. Although the RNase activity of the N was consistently higher, there was no statistically significant difference in RNase activity between 4 d N and M or 6 d N and M seedlings. The activity, however, for both sources declines between 4 and 6 days. Had enzymatic hydrolysis of RNA during isolation posed a significant problem, yields of RNA obtained from 4 d tissue of both M and N seedlings would have probably been reduced to the same extent. Also, SDS-PAGE gels of *in vitro* translation products directed by poly(A) RNA using either wheat germ (Fig. 5, 6) or rabbit reticulocyte lysate (Fig. 7) reveals that high MW products may be synthesized by both cell-free systems and that neither system exhibits an excessive production of low MW degradation products as might be expected if RNase activity was not sufficiently suppressed.

An additional trend seen in Table I is that less mature barley seedlings generally provided better yields of poly(A) RNA. The yield of poly(A) RNA from 7 d old seedlings was so poor that a second passage through oligo(dT)-cellulose became impractical. Consequently, all subsequent analyses were carried out on tissue from 4 to 6 d old seedlings. Higher yields of

Table I. Poly(A) RNA yields from apical leaf segments of 'N and M barley seedlings grown under continuous light (N and M) and under etiolated conditions (E). These values represent single determinations but illustrate trends in the amount of extractable poly(A) RNA between the two sources at the various stages of growth.

Seedling Age (days)	Yield Poly(A) RNA $\mu g \cdot g$ fresh weight	A_{260}/A_{280}
4E N	4.21	1.90
4E M	6.69	1.86
4 N	4.20	1.83
4 M	8.51	1.80
5 N	1.54	2.13
5 M	3.40	2.00
6 N	0.78	1.96
6 M	3.77	1.85

All the above yields were obtained following two cycles of oligo (dT)-cellulose chromatography.

Table II. Total ribonuclease activity in apical leaf segments of 4 and 6 d N and M barley seedlings grown under continuous illumination. Activity expressed in units of activity / min · g fresh wt as suggested by Wilson (235) ± 2 SE. M values also expressed relative to those of N. Each value is based upon data from 4 separate isolations.

Seedling Age	Total RNase Activity	Relative (%)
4N	45.74 ± 4.26	100
4M	37.91 ± 3.68	83
6N	27.38 ± 7.84	100
6M	19.43 ± 5.22	72

Figure 5. Fluorogram of [^{35}S]-methionine labelled wheat germ translation products of poly(A) RNA isolated from 4 d N and M barley apical leaf segments of light-grown and etiolated seedlings. Generally, 0.25-0.5 μg of poly(A) RNA isolated from the appropriate source were used to conduct each *in vitro* translation assay. This figure presents representative electrophoretic profiles obtained following SDS-PAGE on 7.5-15% gradient gels (see Materials and Methods). Each pair, (N and NE, M and ME) were loaded on adjacent lanes with equal cpm/well and subsequently exposed to X-ray film for the same length of time.

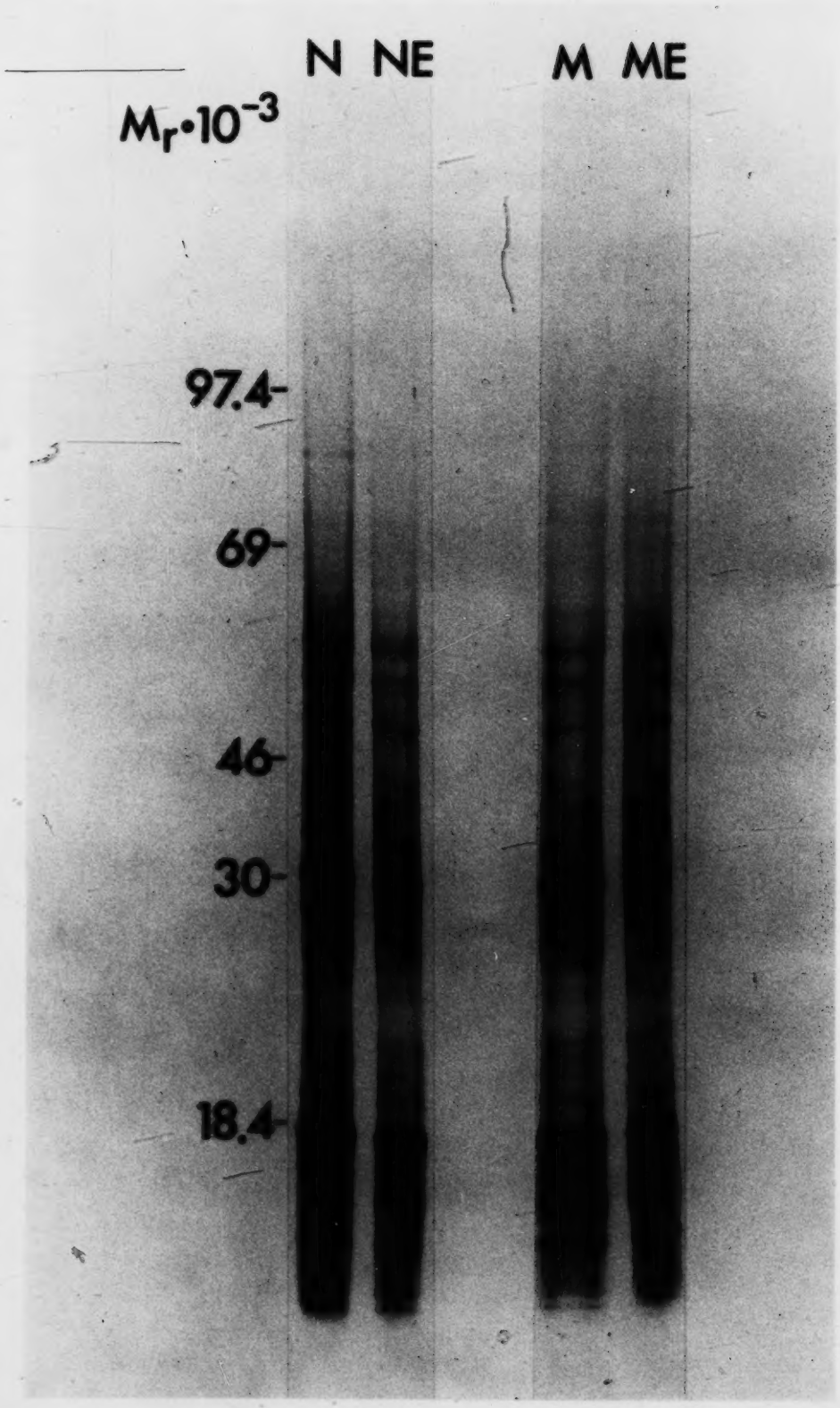

N NE
M ME
$M_r \cdot 10^{-3}$
97.4
69
46
30
18.4

Figure 6. Comparison of [^{35}S]-methionine labelled wheat germ translation products directed by poly(A) RNA isolated from light-grown 4, 5 and 6 d N and M barley apical leaf segments. Barley seedlings grown under continuous illumination for 4, 5 or 6 d were used for the isolation and purification of poly(A) RNA. Generally, 0.25-0.5 μg of poly(A) RNA was used to conduct each *in vitro* translation assay. This figure presents typical electrophoretic profiles obtained following separation of translation products by SDS-PAGE on 7.5-15% gels (see Materials and Methods). Each pair, N and M, corresponding to a specific seedling age represents assay samples loaded on adjacent lanes with equal cpm/well and subsequently exposed to X-ray film for the same length of time.

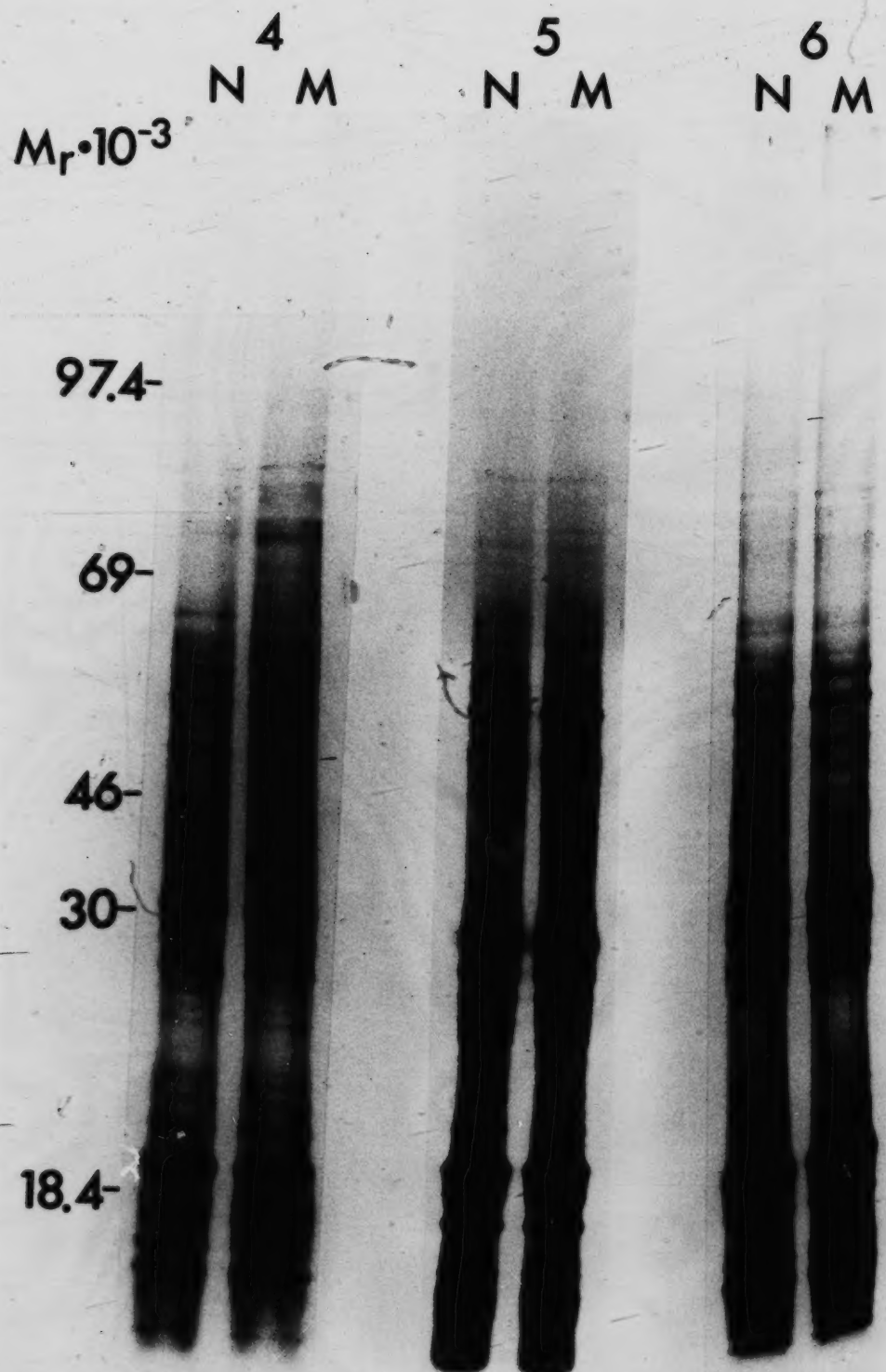
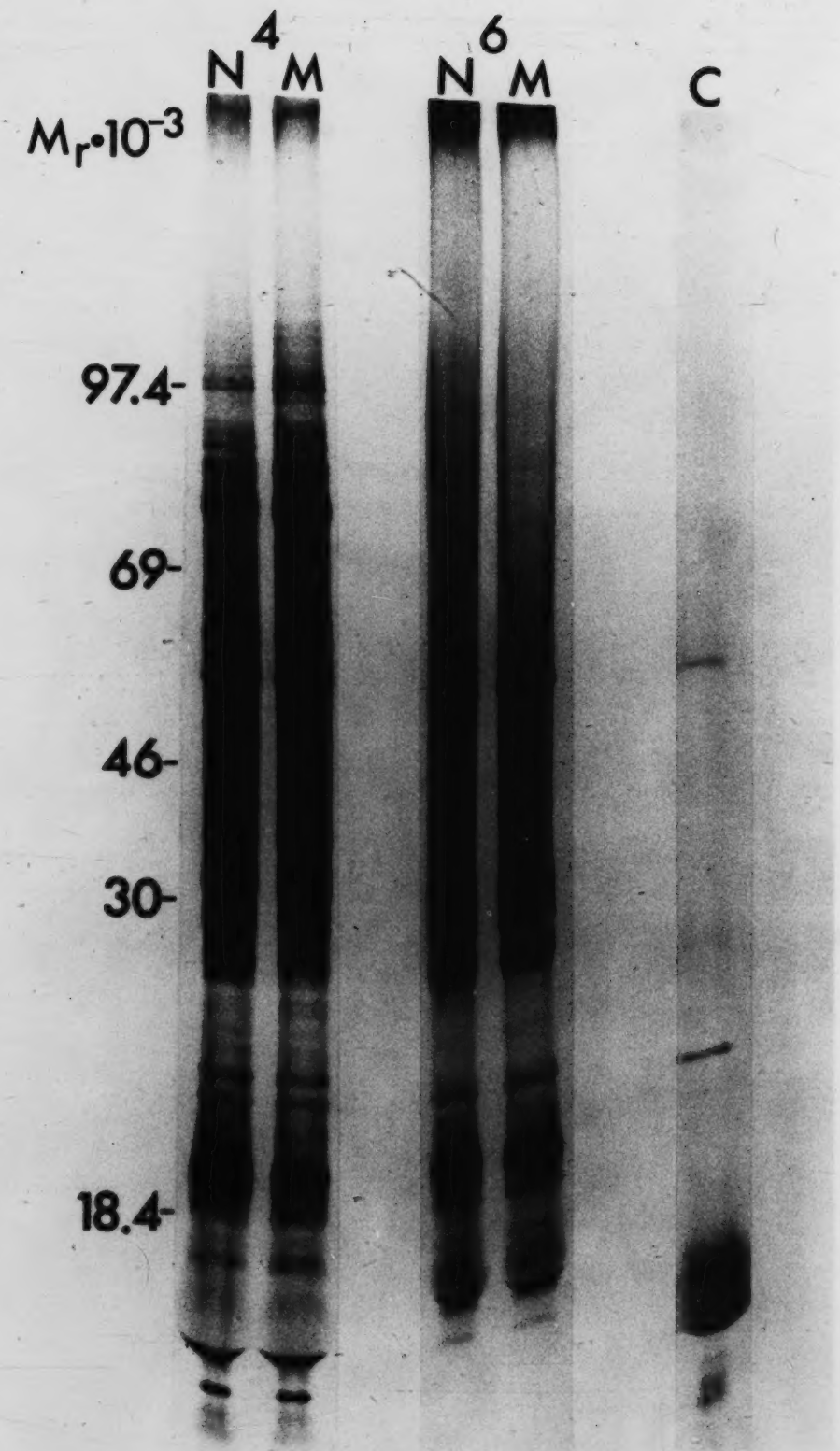

4
N M
5
N M
6
N M
$M_r \cdot 10^{-3}$
97.4-
69-
46-
30-
18.4-

Figure 7. Comparison of [^{35}S]-methionine labelled reticulocyte lysate translation products directed by poly(A) RNA isolated from 4 and 6 d N and M barley apical leaf segments. Barley seedlings grown under continuous illumination for 4 or 6 d were used for the isolation and purification of poly(A) RNA. Generally, 0.25-0.5 μg of poly(A) RNA were used to conduct each *in vitro* translation assay. This figure presents typical electrophoretic profiles obtained following separation of translation products by SDS-PAGE on 7.5-15% gels (see Materials and Methods). Each pair, N and M, corresponding to a specific seedling age represents assay samples loaded on adjacent lanes with equal cpm/well and subsequently exposed to X-ray film for the same length of time. Lane 'C' shows the reticulocyte lysate endogenous translation products.


N 4
M
N 6
M
C
Mr·10-3
97.4-
69-
46-
30-
18.4-

poly(A) RNA has also been reported for the less-differentiated, immature tissue segments of *Zea mays* leaves (133) yet the authors suggest that when yield is expressed on a per cell basis, the converse may actually be true.

The evidence suggests that the M probably has as much polyadenylated RNA as the N at comparable stages of development. The difference observed in RNA content earlier (141) may very well reflect differences in the more abundant RNA classes including rRNA and tRNA.

In vitro Translatability of Poly(A) RNA

When assays were carried out to assess the translatability of poly(A) RNA preparations, both sources provided highly translatable mRNA but only after poly(A) RNA had been subjected to two cycles of oligo (dT)-cellulose chromatography. Table III provides representative values for both [^{35}S]-methionine and [^{3}H]-leucine incorporation into TCA insoluble polypeptides. When assessed for translatability following a single passage through oligo(dT)-cellulose, M poly(A) RNA invariably appeared less translatable *in vitro* particularly when isolated from 4 d-old light or dark-grown seedlings. A second passage through oligo (dT)-cellulose resulted in substantial improvement in the translational activity of both N and M poly(A) RNA preparations. Although much improved, a slight discrepancy is still evident for poly(A) RNA preparations from 4 d etiolated plants (Table III). Although differences in the *in vitro* translatability of poly(A) RNA has not been well documented, Skadsen and Cherry (187) report low translational activity of mRNA isolated from 4 d old soybean cotyledons. They report that the reduced incorporation of [^{3}H]-leucine per μg of added mRNA prevails despite a rather rigorous poly(A) RNA purification procedure. The difference could not be explained by the presence of inhibitors or the less likely possibility of ribosome contamination in the mRNA preparation.

In the case of this study, the observation that the translational discrepancy between the two sources could be alleviated by additional passage through oligo (dT)-cellulose suggests that

Table III. Translational activity of poly(A) RNA isolated from apical leaf segments of N and M barley seedlings. Poly(A) RNA was purified from total RNA by either one or two cycles through oligo (dT)-cellulose (see Materials and Methods) and subsequently translated *in vitro* using a wheat germ system. At the termination of the assay, duplicate aliquots of the reaction mixture were used to determine the incorporation of [^{35}S]-methionine **(A)** or [^{3}H]-leucine **(B)** into TCA-precipitable polypeptides. Each value is representative of at least two determinations and is reported both as absolute cpm and as M incorporation relative to that of the corresponding N ± the maximum percent variation in the cpm obtained for each sample based upon 2 SE of the absolute cpm.

A. Incorporation using [^{35}S]-methionine

Seedling Age (days)	One cycle cpm*	Relative %	Two cycles cpm*	Relative %
4E N	n.d.		396,560	100 ± 4
4E M	n.d.		283,725	72 ± 3
4 N	19,590	100 ± 20	418,846	100 ± 23
4 M	4,811	25 ± 3	391,137	93 ± 4
5 N	13,884	100 ± 9	463,786	100 ± 5
5 M	6,611	44 ± 10	404,191	87 ± 10
6 N	10,012	100 ± 5	418,846	100 ± 23
6 M	10,029	103 ± 11	391,137	93 ± 4

B. Incorporation using [^{3}H]-leucine

Seedling Age (days)	One cycle cpm*	Relative %	Two cycles cpm*	Relative %
4 NE	20,312	100 ± 7	189,465	100 ± 2
4 ME	10,316	51 ± 7	151,452	80 ± 2
4 N	66,059	100 ± 4	224,879	100 ± 2
4 M	12,718	19 ± 1	190,510	85 ± 2
5 N	35,633	100 ± 5	n.d.	
5 M	19,556	55 ± 2	n.d.	
6 N	n.d.		210,586	100 ± 14
6 M	n.d.		216,982	103 ± 10

* cpm /µl assay in a 25 µl assay volume containing 1.0 µg of RNA

a contaminant, likely rRNA, was probably present in the mRNA preparation. In general, rRNA only interferes directly with *in vitro* translation when rRNA content of a preparation is extremely high as might be the case with a total RNA preparation (138,154). However, contamination of this nature could lead to lower rates of incorporation by providing an over-estimate of the amount of translatable mRNA added to an assay. It was also observed that less mature tissue and particularly the M poly(A) RNA preparations were susceptible to these somewhat deceptively low incorporation rates. Possibilities that should be considered include the presence of other UV-absorbing materials including DNA and polysaccharides both of which can exert deleterious effects on *in vitro* translation (153,154). Binding of carbohydrates to oligo (dT)-cellulose has been reported (153). This feature proves particularly troublesome when isolating poly(A) RNA from polysaccharide-rich material.

Translation of Poly(A) RNA

One way of providing a tangible record of mRNA is by translating the messages *in vitro* and analyzing the polypeptide products which are synthesized. It must be acknowledged that the SDS-PAGE separation of polypeptides synthesized *in vitro*, as was done in this study, probably only highlights rather abundant messages or those which may not be particularly abundant but methionine-rich. However, as the M displays a deficiency in proteins which can be considered very abundant including Rubisco (12,106), LHCP (115) and a number of soluble and membrane-associated chloroplast proteins (106) early in development, the patterns of polypeptides synthesized *in vitro* were studied closely to determine whether or not a lag in the appearance of abundant messages might also be observed. An additional limitation is that *in vitro* translation relies upon the capacity of cell-free systems to faithfully translate exogenously added messages. A criticism often raised for wheat germ is that it has a tendency towards premature termination of messages being translated so that long messages are not translated very efficiently (87). Furthermore, some messages are preferentially translated because of their inherent, enhanced translatability in wheat germ and not particularly as a function of their

relative abundance (48). Consequently, a somewhat limited comparison was made for 4 and 6 d material using a reticulocyte lysate cell-free system. The results obtained by both cell-free systems will be dealt with separately.

Translation Products in a Wheat Germ System

Figures 5 and 6 show representative profiles of polypeptides directed by poly(A) RNA isolated from barley apical leaf tissue and translated *in vitro* using a wheat germ cell-free protein synthesis system. The polypeptides, separated by a 7.5-15% SDS PAGE gradient gel, can be resolved into products of MW varying from 12 to over 90 kD.

The first observation made from these gels is the striking similarity which is evident for N and M profiles representative of any of the particular days. Any differences between N and M would appear to be quantitative rather than qualitative. Attention should be drawn to the numerous high MW products which are present in M profiles, as seen in the 4 d M sample of Figure 6, but apparently absent from 4 d N profiles. However, since lanes were loaded with approximately equivalent cpm, the M generally required a greater assay volume to meet this requirement. Similar fluorograms allowed to overexpose suggest that all of these high MW M bands are represented in N profiles.

Figure 5 provides a comparison of polypeptides synthesized by poly(A) RNA isolated from 4 d N and M light-grown and etiolated seedlings. Many of the polypeptides directed by poly(A) RNA isolated from light-grown N seedlings appear to be synthesized at higher levels than similarly migrating products of light-grown M seedlings. The profiles comparing N with NE or M with ME products exhibit analogous increases for a number of polypeptides in response to light but the magnitude of these increases appears to be less pronounced for the M.

A comparison of 4, 5 and 6 d M and N *in vitro* translation products in Figure 6 shows that much of the apparent quantitative variation seen between 4 d M and N has essentially disappeared by 5 and 6 d of development. One translation product of 5 d material which only appears on over-exposed fluorograms and therefore cannot be seen in Figure 6 migrates with an

apparent MW of 113 kD and does not appear to be as well represented among M products as compared to those of the N. Assignment of this MW is rather tentative as it lies outside the range of MW standards. However, as polypeptides in this range are not efficiently synthesized by wheat germ, the failure of a large product to accumulate quantitatively may not represent a difference in mRNA abundance. The 6 d profiles do not reveal any discernible differences.

When Figure 6 is viewed as a function of development, shifts in the relative abundance of some products is suggested. One product that seems to undergo an increase is the polypeptide of 53 kD. Levels may also be enhanced for messages encoding the two closely-migrating polypeptides of 32 and 33 kD in addition to one of approximately 19 kD. As all of these products are synthesized by poly(A) RNA the messages are probably nuclear-encoded.

Translation Products Synthesized in a Reticulocyte Lysate System

The array of polypeptides synthesized by the reticulocyte lysate cell-free system and resolved by SDS-PAGE (Fig. 7) bears little resemblance to those produced by wheat germ (Fig. 6). For example, the wheat germ products that appear to migrate as a dimer of MW 32 and 33 kD are presumably identical to similarly migrating polypeptides which are only very weakly displayed relative to the other products synthesized by the reticulocyte lysate. Their presence becomes more apparent among the polypeptides directed by 6 d N and M mRNA.

The products synthesized by this system ranged from 13 to over 100 kD. Comparisons made between 4 and 6 d N and M polypeptide profiles reiterates the striking qualitative similarities seen with the wheat germ results. However, two quantitative differences which can be noted among 4 d N and M products include decreased synthesis of 27 and 19 kD M polypeptides relative to those of the N. By 6 d, however, even these slight differences are no longer evident.

With respect to changes related to development, polypeptides in the range of 27 to 33 kD undergo substantial increases. A very prominent 53 kD product seen at 4 d remains equally well represented among 6 d polypeptides. Analogous shifts from 4 to 6 d poly(A) RNA

directed polypeptides are seen for both the N and M.

C. Polysome Profile Analysis

Assessment of Translational Capacity

The studies using poly(A) RNA suggests that the M can produce substantial levels of translatable mRNA which encodes a diverse range of polypeptides. The second objective of this study was undertaken to assess the capacity of the M to carry out protein synthesis *in vivo*.

Mösinger and Schopfer (148) suggest that translational capacity can be estimated using polysomal profiles generated from extracts of plant material layered directly onto sucrose gradients. Following centrifugation, the absorbance profile of eluted gradients can be used to determine the ratio of polysomes to ribosomal monosomes and subunits. The authors state that this ratio provides a quantitative estimate of *in vivo* translational ability.

A drawback to assessments of this nature is the general lack of agreement between many authors regarding how and when such estimates can be made. As a consequence, comparison between the reported values of different laboratories can become a tenuous proposition. For example, Davies and Larkins (54) divided profiles into three areas designated large polysomes (LP, $>$ 5mers), small polysomes (SP, $<$ 5mers) and monosomes (M) and using these areas, computed ratios of $LP/LP+SP$ and $M/LP+SP+M$. However, Davies (53) reported the actual areas associated with each division as opposed to ratios. Other groups present ratios of polysomes to monosomes (77), the frequency of polysomal size classes (190), or any of a number of permutations.

For the purpose of this study, the recommendations of Mösinger and Schopfer (148) were followed. Basically, polysome profiles were divided by a vertical line into two regions including one associated with active translation namely the polysomes (P) and the fraction not involved with active protein synthesis composed of ribosomal monomers and subunits ($M + S$). The ratio of the two regions, or $P:M + S$, was computed for profiles generated by 4

and 6 d N and M preparations. Table IV shows that the ratios of $P:M + S$ for 4 d N and M samples are significantly different and similar ratios for 6 d N and M preparations are highly significantly different.

This would suggest, based upon quantitative criteria, that the M has a greater *in vivo* translational capacity than the N. However, this assumption is somewhat misleading. Figure 8 demonstrates that though the area represented by P is basically very similar for both N and M, the absolute area occupied by $M + S$ is much reduced in M profiles as compared to those of the N. Therefore, the denominator used in the calculations for N samples is much larger leading to a smaller $P:M + S$ value. If the assumption that the upper part of the profile is composed primarily of 80S and 70S ribosomes in addition to their respective subunits is valid, a reduced quantity of these components for the M is implied by this analysis. When the region of the profile, P, is expressed as a proportion of the total profile determined by $P + M + S$, polysomes occupy 51% of N and 56% of M polysomal profiles. These percentages drop by 6 d to 32% and 43% for N and M, respectively.

Qualitatively, however, a major difference in the shape of the profiles generated by the two sources is readily apparent even in those obtained from 4 d etiolated plants (Fig. 10). Although Figure 10 shows that etiolated material exhibits very little evidence of polysome formation, a feature reported for other dark-grown seedlings (74,76), a distinctive peak is found in profiles of 4 d N etiolated samples which is notably absent in that of the corresponding M. Feierabend and Schrader-Reichardt (72) identify a similar peak which lies adjacent to the 80S peak in profiles generated from rye seedlings as indicative of 70S ribosomes. This peak is more clearly seen positioned as a shoulder on the 80S monosome peak in Figure 11 which presents similar samples obtained from etiolated N and M seedlings. For this figure the sucrose gradients were eluted and absorbance recorded at a higher sensitivity. A shoulder is also apparent in profiles of 4 d N seedlings grown under continuous illumination (Fig. 8A) but is absent or at least very much reduced in the corresponding M profile (Fig. 8B). Profiles obtained from 6 d samples reveal a large, broad peak associated with the N sample (Fig. 9)

Table IV. Analysis of polyribosome distribution in 4 and 6 d N and M barley apical leaf segments. Polysomal profiles depicted in Figures 8 and 9 were separated into two sections by drawing a vertical line between the 80S monosome peak and the polysome region. The area to the left of the line was designated polysomes (*P*) while all peaks to the right of the division were assumed to be composed of monosomes (80S and 70S) (*M*) and ribosomal subunits (*S*) (148). The ratio of the two areas was determined by tracing the respective regions onto weighing paper and employing the ratio of the two weights obtained. For the purpose of area determination, only the profile extending above the baseline absorbance was considered. For comparison, the polysome region is also expressed as a ratio of the total area composed of $P+M+S$.

Seedling age (days)	$P/P+M+S$	$P/M+S$
4 N	0.512 ± 0.025	1.054 ± 0.108
4 M	0.559 ± 0.033	1.278 ± 0.160
6 N	0.324 ± 0.030	0.482 ± 0.060
6 M	0.428 ± 0.050	0.760 ± 0.160

Values represent the mean of 4 different polysome gradients ± 2 SE.

which is very much reduced in size in profiles of the M. The identity of this peak was not determined yet its position suggests that at least part of it is composed of 70S ribosomes. Mösinger and Schopfer identify a comparable, similarly migrating peak as the large subunit (60S) of cytoplasmic 80S ribosomes. However, the two smaller peaks which are positioned closer to the top of the sucrose gradient in Figures 8 and 9 are more likely to be the large (60S) and small (40S) subunits of 80S ribosomes. The possibility that this peak is associated with the degradation of polysomes during isolation cannot be excluded. However, considerations that identical methods provided well-resolved profiles of 4 d polysomal gradients, profiles for 6 d N were very reproducible, the size of the 80S monosome peak did not increase as is generally observed when polysome preparations are treated with RNase (118,134) and the relatively low endogenous ribonuclease activity of 6 d M and N apical leaf tissue (Table III) all suggest that enhanced degradation of polysomes does not explain the presence of the peak highlighted by the arrow.

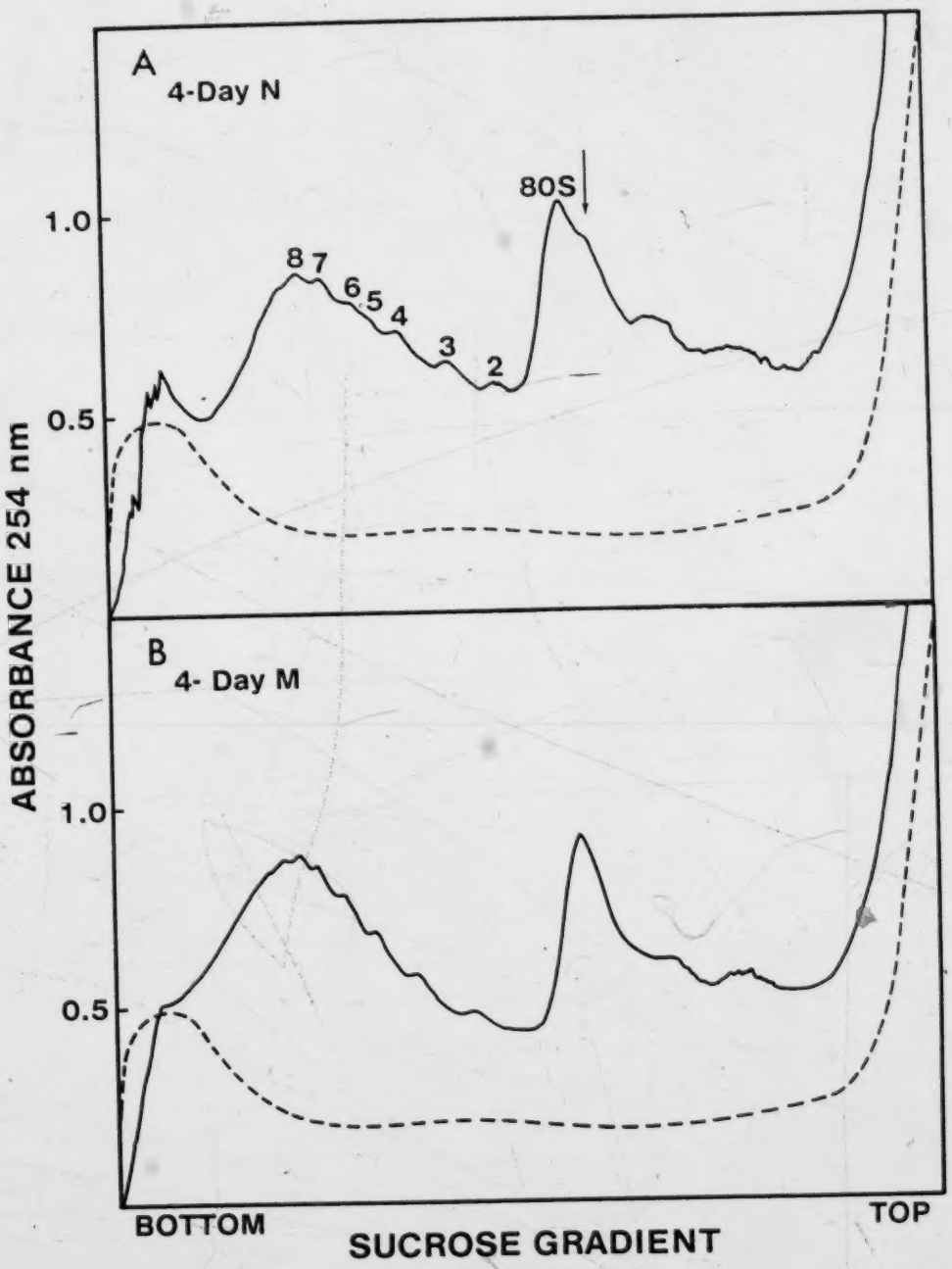


Figure 8. Sucrose gradient profiles of ribosomal extracts of 4 d N (A) and M (B) barley apical leaf segments prepared from seedlings grown under continuous illumination. Extraction procedures were carried out to include both membrane-bound and cytosolic ribosomal fractions. The numbers in A refer to the polysomal size classes, 80S designates the position of the major cytoplasmic monosomal peak while the arrow highlights the position of 70S ribosomes. The dashed line represents the baseline absorbance of gradients overlayed with extraction buffer. Profiles are representative of four separate experiments.

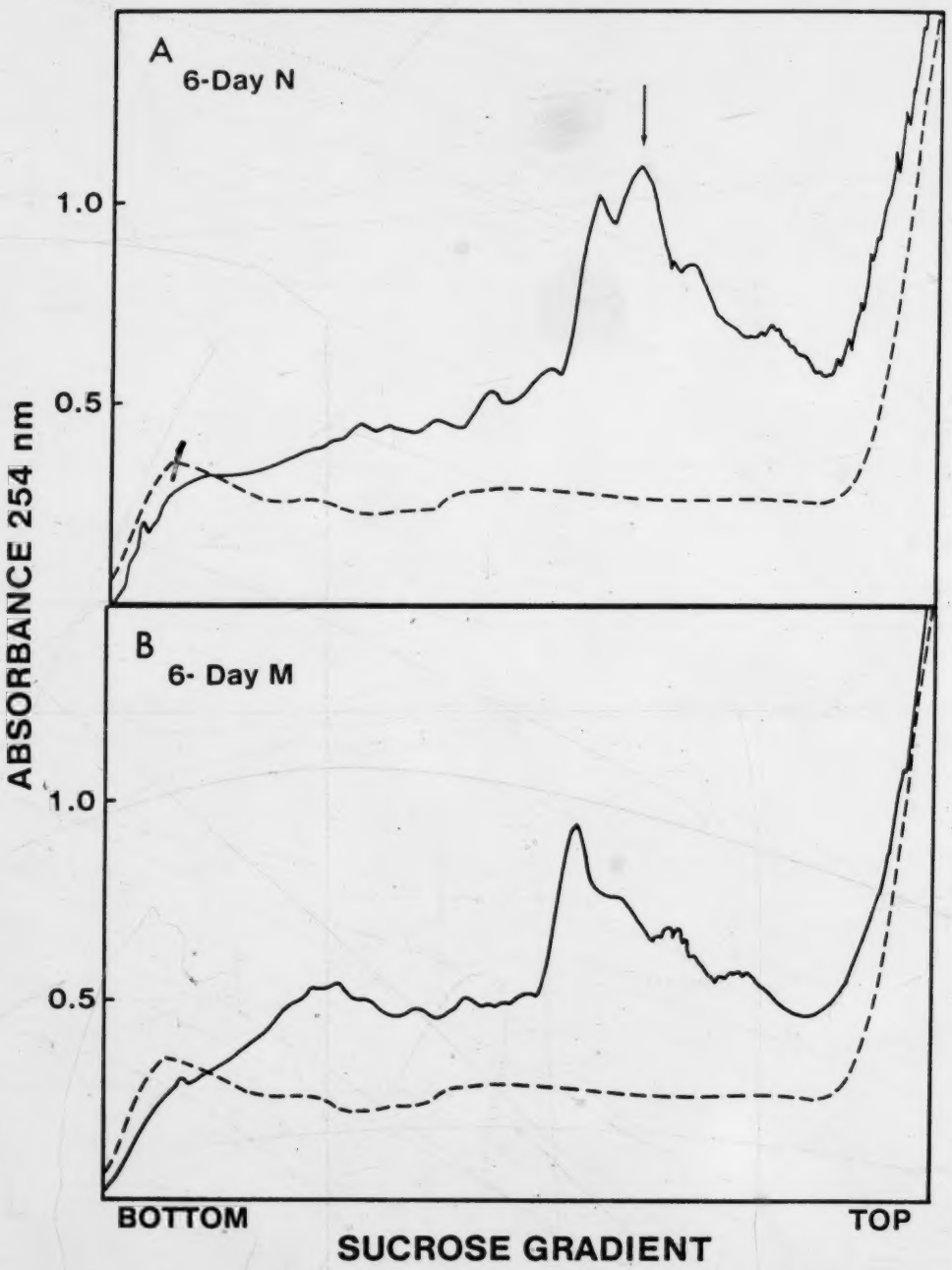


Figure 9. Sucrose gradient profiles of ribosomal extracts of 6 d N (A) and M (B) barley apical leaf segments prepared from seedlings grown under continuous illumination. Ribosomal extracts including membrane-bound and cytosolic ribosomal fractions were layered directly onto sucrose density gradients. The arrow highlights the position of a prominent peak associated with 70S ribosomes. The dashed line indicates the baseline absorbance of gradients overlayed with extraction buffer. Profiles are representative of three separate experiments.

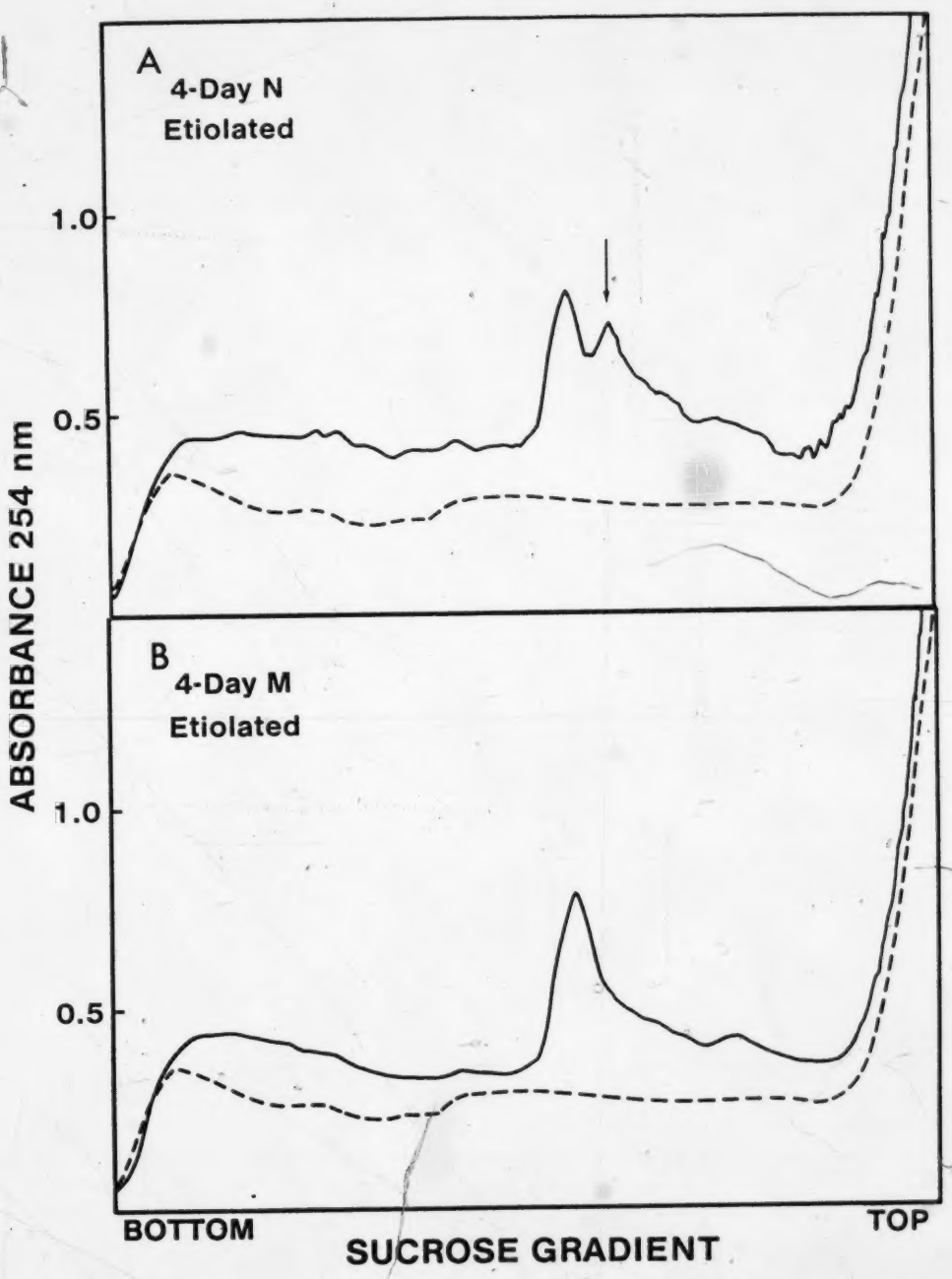
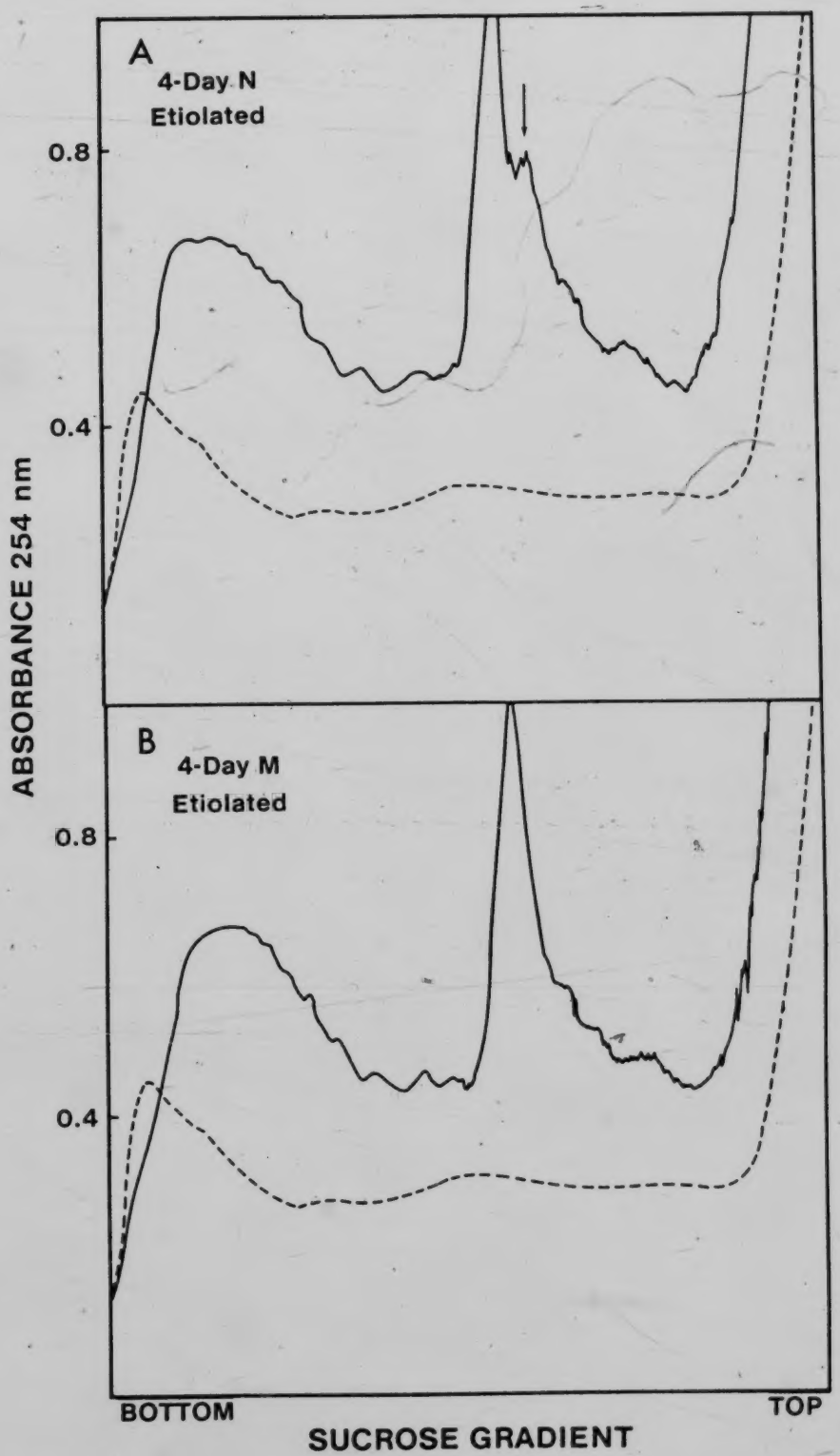


Figure 10. Sucrose gradient profiles of ribosomal extracts of 4 d N (A) and M (B) barley apical leaf segments prepared from etiolated seedlings. Ribosomal extracts including membrane-bound and cytosolic ribosomal fractions were layered directly onto sucrose density gradients. The arrow designates the position of the 70S monosome peak adjacent to the more-pronounced 80S monosome peak. The dashed line indicates the baseline absorbance of gradients overlayed with extraction buffer. Profiles are representative of three separate experiments.

Figure 11. Ribosomal extracts of 4 d etiolated N (A) and M (B) barley seedlings prepared to highlight the position of 70S ribosomes. Elution of sucrose density gradients was carried out and the absorbance was recorded at a higher sensitivity than the profile depicted in Figure 10. Otherwise, the conditions and description of the profile are identical to those of Figure 10.


A
4-Day N
Etiolated
0.8
0.4
B
4-Day M
Etiolated
0.8
0.4
ABSORBANCE 254 nm
BOTTOM
TOP
SUCROSE GRADIENT

The discrepancy seen in the M as compared to the N polysomal profiles depicted in Figures 8 to 11 is probably best explained by a markedly reduced quantity of 70S ribosomes in the M. Reduced yields of 70S ribosomes from this M have already been reported in a previous study from this laboratory (114). Also, a study of plastid development using this M (107) revealed that etioplasts of the M do not contain prolamellar bodies. More recently, Wellburn *et al.* (226) have examined prolamellar bodies of *Avena sativa* and *Hordeum vulgare* and demonstrated that these bodies are associated with ribosome-like particles. However, in contrast to the *tigrina* o^{34} mutant of barley which undergoes a disintegration of preformed 70S ribosomes when exposed to non-permissive temperatures, this *virescens* mutant appears to accumulate this class of monosomes as it develops (see Fig. 8 and 9).

RNA Analysis

In order to characterize the translatable mRNAs associated with polysomes, total RNA was isolated from each gradient fraction and an aliquot removed for *in vitro* translation using a wheat germ cell-free system. As the seedlings are phenotypically most distinct at 4 d, attention was directed principally towards gradients generated from 4 d N and M apical leaf segments.

The RNA extracted from polysomal gradient fractions proved to be very active in directing protein synthesis *in vitro* (Fig. 12). As expected, fractions associated with the bottom of the gradient, which corresponds to the polysomal region of the gradient in Figure 8, are most active in incorporating [^{35}S]-methionine into TCA-insoluble polypeptides. The fractions which show poor incorporation (fractions 13-16) correspond roughly to the position of the monomers of Figure 8. These fractions would be enriched with rRNA and would not translate very efficiently. The slight increase in incorporation seen in fractions 17 to 23 associated with the monomers and subunits is probably due to mRNA associated with ribonucleoproteins other than ribosomes and, hence, these messages are probably not being actively translated by the plant. Evidence that polypeptides are indeed being synthesized is provided by Figures 13 and 14 which show products synthesized *in vitro* by RNA extracted from 4 d N and M polysomal

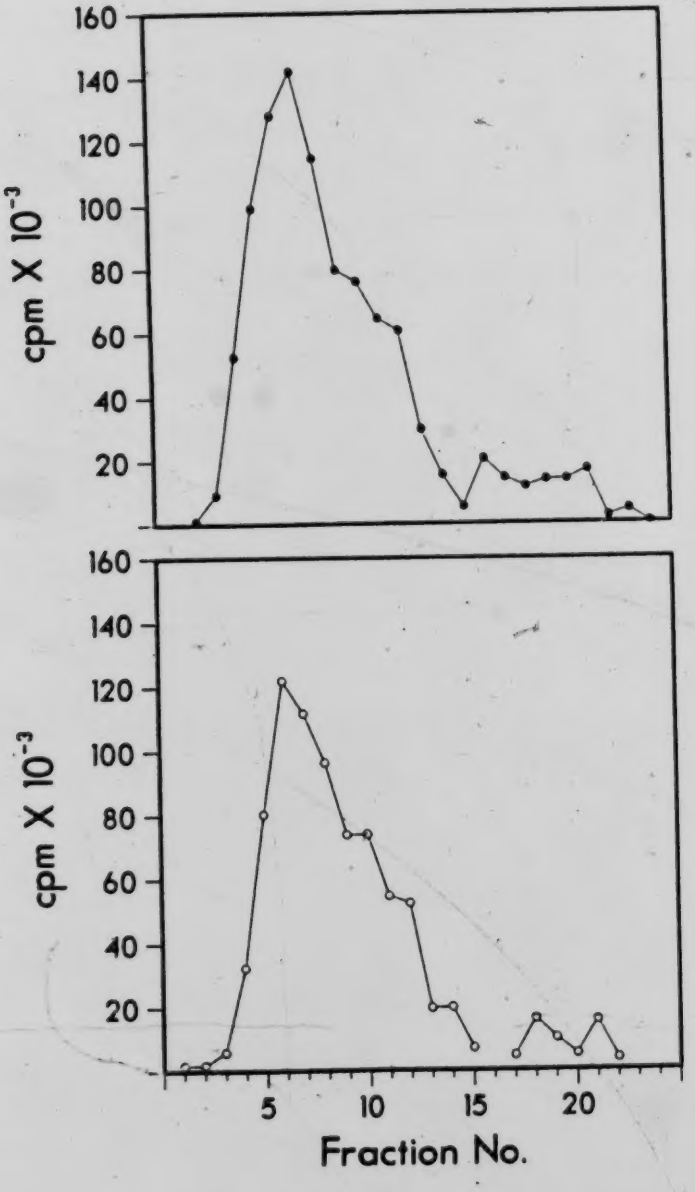


Figure 12. *In vitro* translatability of RNA extracted from 4 d N (●) and M (○) polysomal gradients. An arbitrary 3 μl aliquot of total RNA extracted from each polysomal gradient fraction was translated *in vitro* using a wheat germ system (see Materials and Methods). The total assay volume was 12.5 μl. At the termination of the assay, duplicate 1 μl aliquots were withdrawn and used to determine [^{35}S]-methionine incorporation into TCA-precipitable polypeptides. Each value on the graph has been corrected for incorporation by the wheat germ control assay and negative values are not shown. Assays for each sample were repeated 3 times. Fractions are numbered from the bottom to the top of the gradient.

gradient fractions. These profiles provided a somewhat simplified array of translated products and permitted an easier comparison to be made between the polypeptides synthesized by RNA isolated from the two sources. Differences in polypeptides in the range between 18-30 kD and 46-60 kD are apparent. As Figure 12 suggests, mRNA associated with fractions corresponding to the polysomes (lanes 5 to 10, Fig. 13 and 14) encode a wide variety of polypeptides. Messages for some polypeptides appear to be well represented in a number of polysomal size classes while others are not as well distributed in the profile. One 12 kD polypeptide, however, appears to be found almost exclusively in fractions near the top of the gradient profile (fraction 17) and is presumably associated with ribonucleoproteins. However, as critical alignment of banding patterns on different gels is not possible, polysomal RNA was used for additional analyses in both wheat germ and rabbit reticulocyte lysate cell-free assay systems.

In the initial assays used to characterize the polysomal RNA, fractions which were most active in incorporating [^{35}S]-methionine (as determined by incorporation curves depicted in Fig. 12) were used for *in vitro* synthesis. When these "peak" fractions from N and M polysomes were compared using reticulocyte lysate (Fig. 15A) at least 8 N polypeptide products (lane 4) exhibited poor or no visible counterpart in the adjacent lane of M products (lane 5). Polypeptides corresponding to apparent MW values of approximately 56, 47, 40, 32, 28, 21.5, 19 and 17 kD exhibited the most dramatic differences. The flanking lanes, 3 and 6, are 4 d N and M poly(A) RNA-directed products and are provided as a comparison. A bit disconcerting, however, is the wheat germ equivalent of this fluorogram presented in Figure 16. Depicted by this fluorogram is the remarkable agreement between the polypeptides encoded by N and M polysomal RNA seen in lanes 4 and 5, respectively. Few, if any, variations can be seen. One polypeptide migrating with an apparent MW of about 53 kD is not represented well among M polysome products and a reduced intensity of the 19 kD band is also observed. The presence of high MW bands in lane 5 that are not visible in lane 4 is probably a function of loading. That is, as equal cpm were loaded in each well, a larger assay aliquot of M products was needed to fulfill this requirement and therefore this does not suggest a real difference between N and M

Figure 13. *In vitro* translation products directed by total RNA isolated from fractions of 4 d N polysomal gradients. Total RNA isolated from each polysomal gradient fraction was translated *in vitro* using a wheat germ system. For [^{35}S]-methionine labelled product analysis, 5 μl of each reaction mixture was added to 15 μl of solubilizing buffer, heated and the entire volume was loaded onto 7.5-15% SDS-PAGE gels (see Materials and Methods). Gels were dried down and exposed to X-ray film for 4 d. 'C' designates the control, 0 μg RNA lane. The numbers listed above the lanes refer to the gradient fractions. Lanes 1 to 24 represent fractions collected from the bottom to the top of the polysome sucrose gradient (see Fig. 8A).

$M_r \cdot 10^{-3}$
C
97.4
69
46
30
18.4

Figure 14. *In vitro* translation products directed by total RNA isolated from fractions of 4 d M polysomal gradients. Total RNA isolated from each polysomal gradient fraction was translated *in vitro* using a wheat germ system. For [^{35}S]-methionine labelled product analysis, 5 μl of each reaction mixture was added to 15 μl of sample solubilizing buffer, heated and the entire volume loaded onto 7.5-15% SDS-PAGE gels (see Materials and Methods). Gels were dried down and exposed to X-ray film for 4 d. 'C' designates the control, 0 μg RNA lane. The numbers listed above the lanes refer to the gradient fractions. Lanes 1 to 23 represent fractions collected from the bottom to the top of the polysome sucrose gradient (see Fig. 8B).

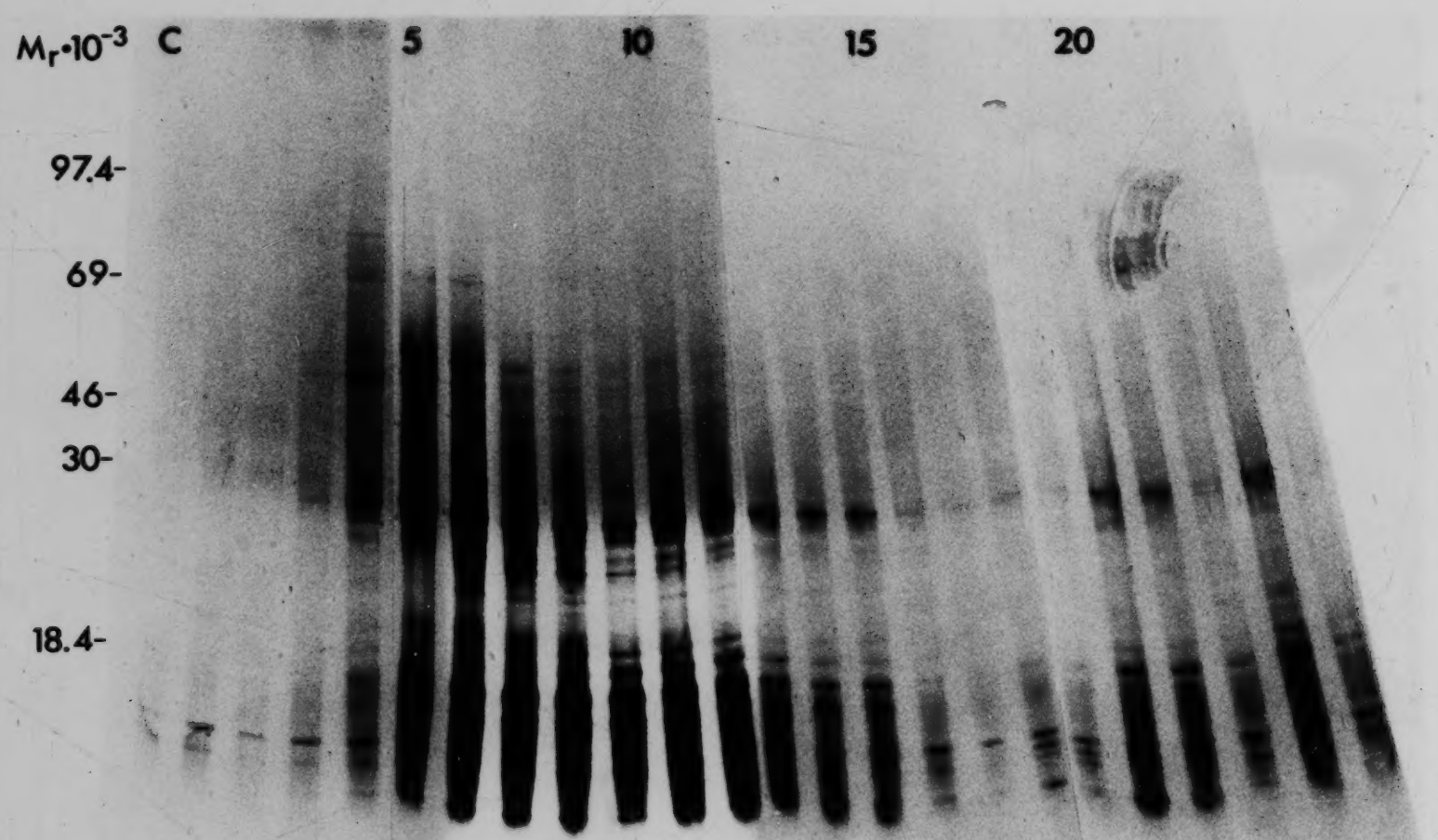

$M_r \cdot 10^{-3}$
C
5
10
15
20
97.4-
69-
46-
30-
18.4-

Figure 15. Fluorogram of reticulocyte lysate translation products of poly(A) RNA and polysomal RNA from 4 d N and M light-grown seedlings. Lanes 1 and 3 are 4 d N poly(A) RNA products while lanes 2 and 6 are 4 d M poly(A) RNA products. Lane 4 is N polysomal RNA from fraction 7 of a polysomal gradient (see Fig. 8A and 12) and lane 5 exhibits products synthesized by M polysomal RNA from fraction 6 of a polysomal gradient (see Fig. 8B and 12). Lane 7 was loaded with endogenous reticulocyte lysate products. Lanes 1 through 6 were loaded with 500,000 cpm of [^{35}S]-methionine-labelled products and lane 7 with 180,000 cpm. The fluorogram was exposed for 4 d. The MW values corresponding to the numbers positioned between lanes 4 and 5 are as follows: 56 kD(1), 47 kD(2), 40 kD(3), 32 kD(4), 28 kD(5), 21.5 kD(6), 19 kD(7) and 17 kD(8).

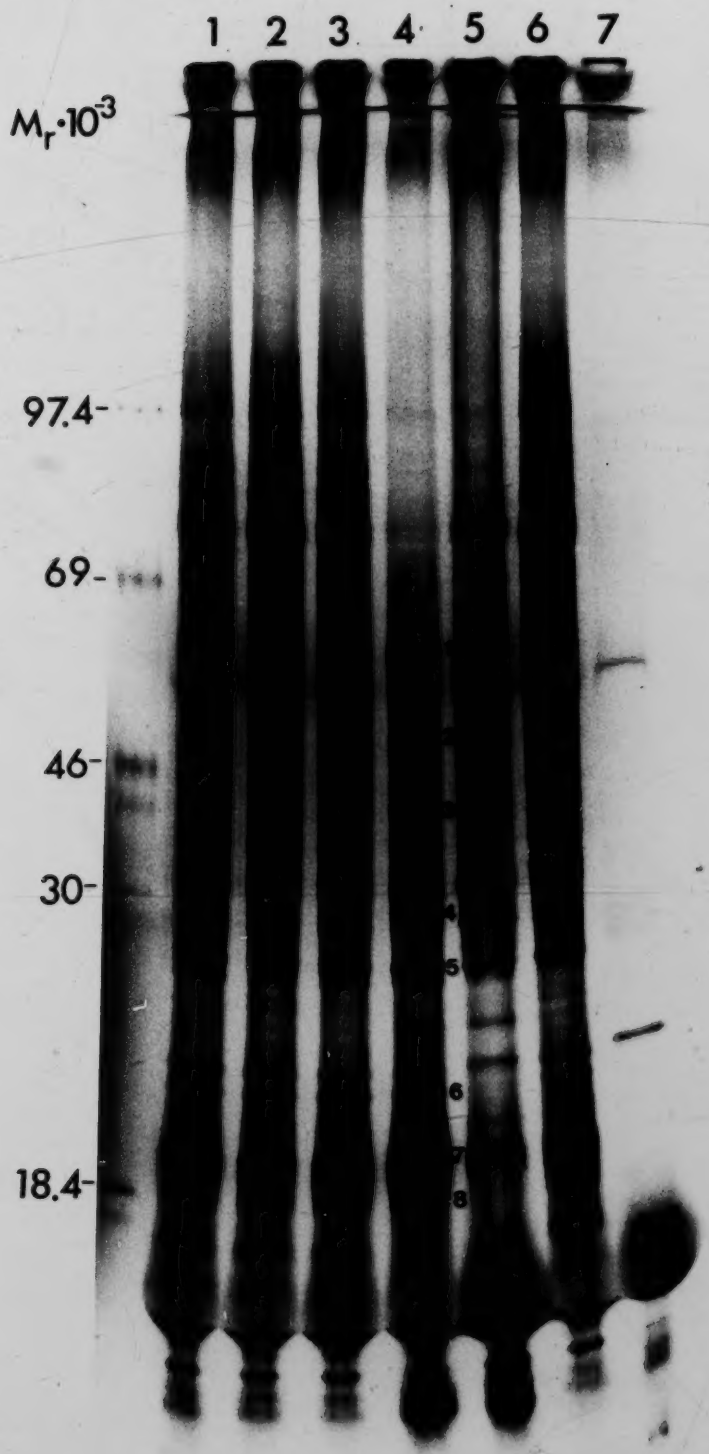
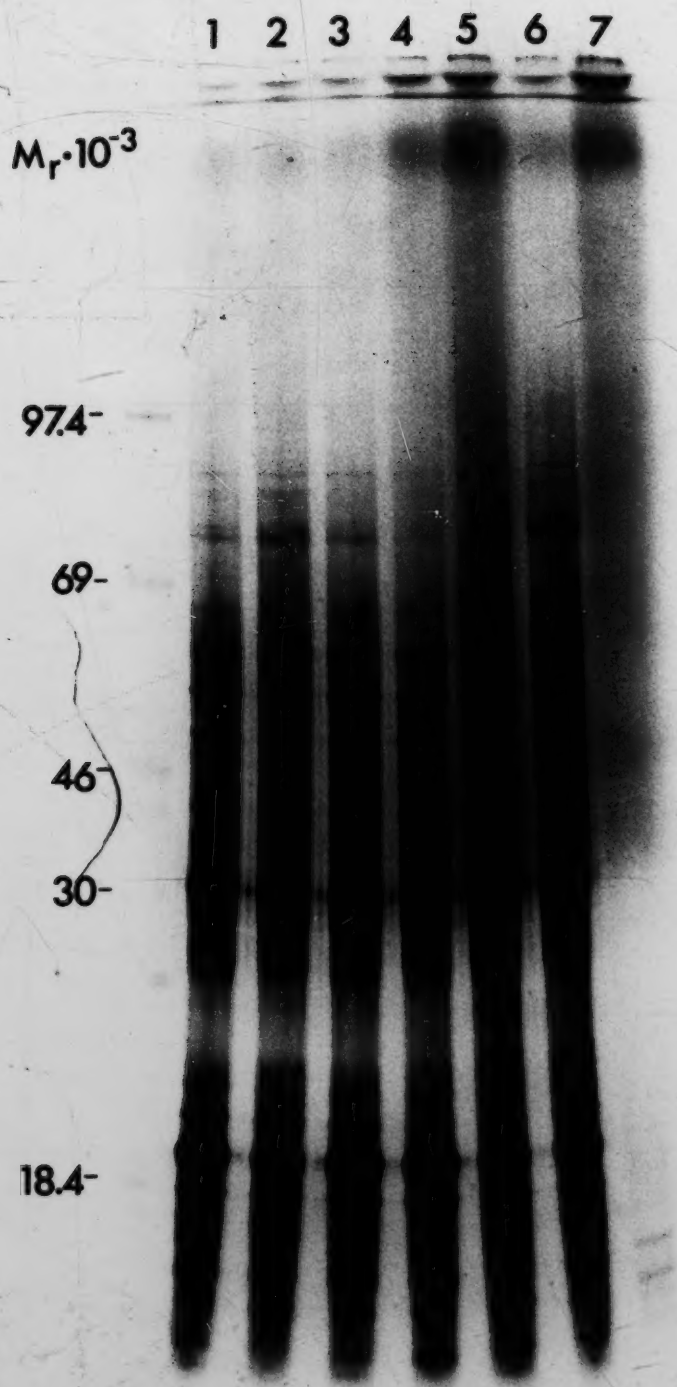

1 2 3 4 5 6 7
$M_r \cdot 10^{-3}$
97.4-
69-
46-
30-
18.4-
4
5
6
7
8

Figure 16. Fluorogram of wheat germ translation products of poly(A) RNA and polysomal RNA from 4 d N and M light-grown seedlings. Lanes 1 and 3 are 4 d N poly(A) RNA products while lanes 2 and 6 are 4 d M poly(A) RNA products. Lane 4 shows products synthesized by 4 d polysomal RNA extracted from fraction 7 of a polysomal gradient (see Fig 8A. and 12) and lane 5 exhibits products directed by polysomal RNA extracted from fraction 6 of a polysomal gradient (see Fig. 8B and 12). Lanes 1 to 6 were loaded with 500,000 cpm of [^{35}S]-methionine-labelled products and lane 7 with 86,000 cpm. The fluorogram was exposed for 4 d.


1 2 3 4 5 6 7
$M_r \cdot 10^{-3}$
97.4-
69-
46-
30-
18.4-

products in the range. This explanation would also apply to Figure 15.

As the assumption that one selected fraction might be representative of an entire profile is not likely valid, similar polysomal RNA fractions were pooled and used for *in vitro* translation. The results of these assays are presented in Figure 17 for reticulocyte lysate and Figure 18 for wheat germ. Comparison of lanes 3 and 6 in Figure 17 (representing N and M polysomal products respectively), shows that the mRNA encoding the major polypeptide of 56 kD, which is also present in lane 4 of Figure 15, is either absent or only very weakly resolvable among M products. For both N and M, this polypeptide is not synthesized by RNA isolated from polysomes of 4 d etiolated seedlings (lane 4, N and 7, M). Otherwise, the numerous, rather marked differences seen in Figure 15, have become far less pronounced. The comparable wheat germ fluorogram (Fig. 18) does not exhibit a similarly migrating 56 kD product nor are any variations suggested by the products directed by polysomal RNA of 4 d light-grown seedlings (lanes 3 and 6 for N and M, respectively). Figures 17 and 18 also show that RNA isolated from polysomes of 4 d etiolated N (lane 4, Fig. 17 and 18) seedlings direct the synthesis of very few polypeptides. It is apparent that the reticulocyte lysate products (lanes 4 and 7, Fig. 17) are primarily endogenous polypeptides. Light induces increases in the synthesis of a number of polypeptides as seen for reticulocyte lysate (Fig. 17) and wheat germ (Fig. 18). Furthermore, many of these polypeptides which undergo enhanced synthesis are represented among poly(A) RNA directed products (lanes 2 and 5, Fig. 17 and 18) and, therefore, suggest a nuclear origin.

A number of characteristics provide a tentative identification for the 56 kD polypeptide. As discussed earlier, the popular heterologous cell-free protein synthesizing systems including those derived from *E. coli*, wheat germ and rabbit reticulocyte lysate cells all exhibit varying efficiencies towards translating mRNAs. A property of wheat germ that has been reported a number of times includes its apparent failure to recognize and respond to exogenously added chloroplast mRNA (26,48). The failure to synthesize the very abundant polypeptide encoded by Rubisco LSU mRNA using wheat germ has been noted in numerous

Figure 17. Fluorogram of [^{35}S]-methionine labelled reticulocyte lysate assay products directed by polysomal RNA isolated from 4 d N and M light-grown and etiolated seedlings. Total RNA was isolated from fractions collected from sucrose density gradients (see Fig. 8, 10 and 12). Fractions corresponding to polysomes were pooled and used for an *in vitro* translation assay. Lanes 2 and 5 contain translation products directed by poly(A) RNA from the N and M, respectively. Lanes 3 to 7 contain polypeptides directed by polysomal RNA pooled from 4 d N (lane 3), 4 d M (lane 6), etiolated 4 d N (lane 4) and etiolated 4 d M (lane 7). Lanes 2 to 7 are loaded with equivalent cpm. Lane 1 was loaded with endogenous reticulocyte lysate products. Fluorogram was exposed for 4 d. The arrow designates the position of a 56 kD polypeptide.

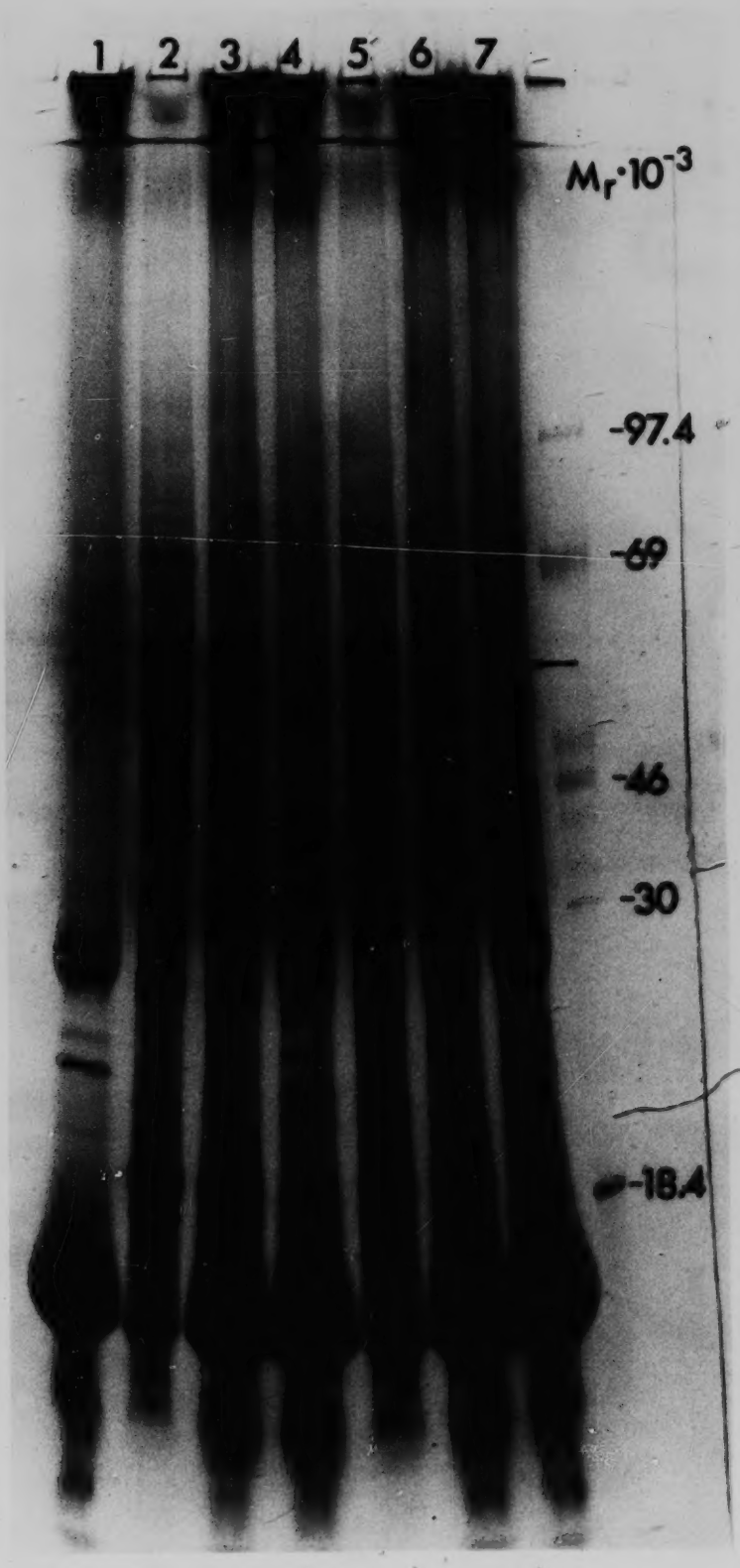
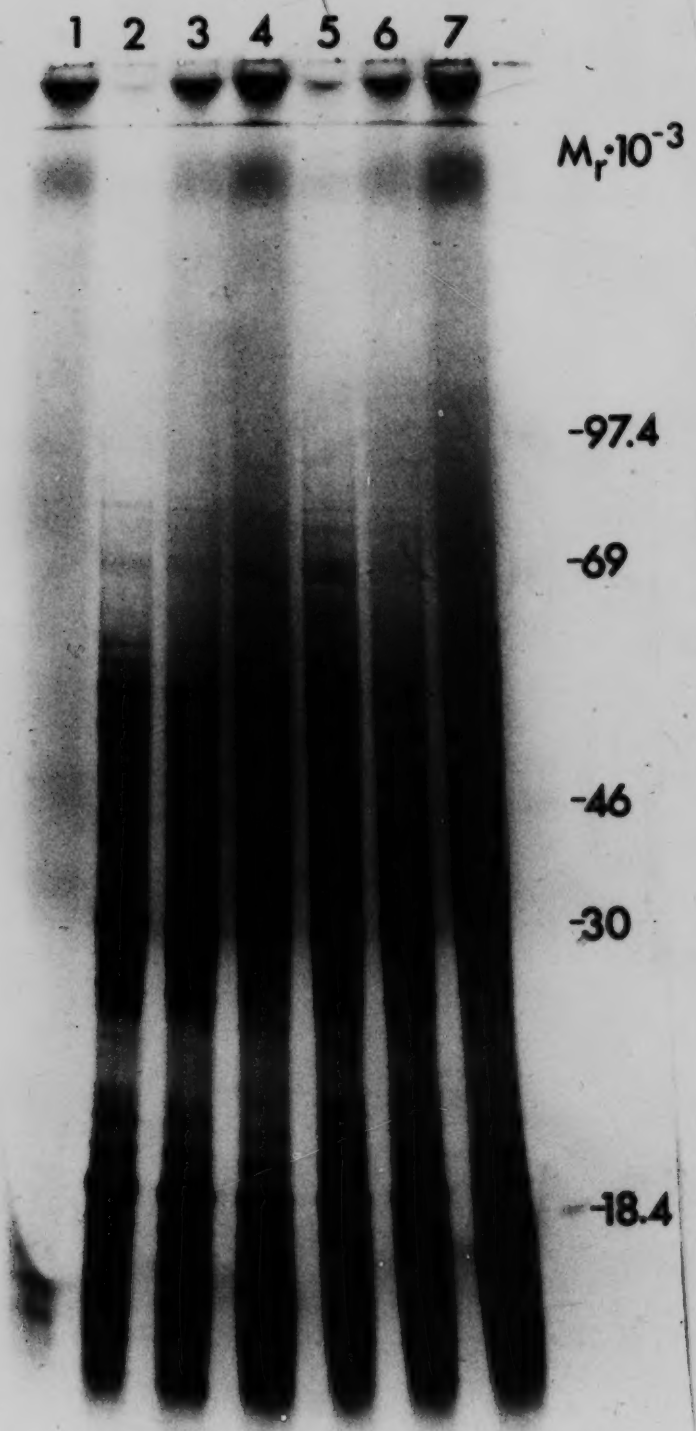

1
2
3
4
5
6
7
$M_r \cdot 10^{-3}$
-97.4
-69
-46
-30
-18.4

Figure 18. Fluorogram of [^{35}S]-methionine labelled wheat germ assay products directed by polysomal RNA isolated from 4 d N and M light-grown and etiolated seedlings. Total RNA was isolated from fractions collected from sucrose density gradients (see Fig. 8, 10 and 12). Fractions corresponding to polysomes were pooled and used for an *in vitro* translation assay. Lanes 2 and 5 contain translation products directed by poly(A) RNA from the N and M, respectively. Lanes 3 to 7 contain polypeptides directed by polysomal RNA pooled from 4 d N (lane 3), 4 d M (lane 6), etiolated 4 d N (lane 4) and etiolated 4 d M (lane 7). Lanes 2 to 7 are loaded with equivalent cpm. Lane 1 was loaded with endogenous wheat germ products. The fluorogram was exposed for 4 d.


1 2 3 4 5 6 7
$M_r \cdot 10^{-3}$
-97.4
-69
-46
-30
-18.4

studies including those using RNA from spinach (26,35), *Chlamydomonas reinhardtii* (98) and wheat (193). Coen (48) suggests that this failure cannot be overcome by manipulating the ionic concentration of the assay medium, a technique that is often used to enhance selective mRNA translation. However, successful use of wheat germ for this purpose has been reported for *Euglena* LSU (171). In fact, Reisfeld and Edelman (161) recommend the use of wheat germ specifically for the *in vitro* translation of chloroplast poly A$^-$ RNA from *Euglena*, *Spirodela* and *Nicotiana* although they acknowledge that the efficiency of the system towards specific mRNAs may fluctuate with assay conditions.

In this study, immunoprecipitation of wheat germ *in vitro* translation products (Fig. 19, N and Fig. 20, M) using antibody raised against Rubisco holoenzyme failed to immunoprecipitate any product migrating in the MW range of the LSU. However, Figure 19 shows that the precursor (pSSU) was readily identified among wheat germ products directed by 4 d N poly(A) RNA (Fig. 19, lane 3) and polysomal RNA (Fig. 19, lane 6) in addition to 4 d M poly(A) RNA (Fig. 20, lane 3) and polysomal RNA (Fig. 20, lane 6). For comparison, reticulocyte products are also shown. With respect to the lysate products in the fluorogram of Figure 19, lane 4, the band at 56 kD is clearly shown despite a very short term exposure (one day) of the gel to X-ray film. No comparable band is found among M lysate products in Figure 20, lane 4 despite a longer exposure period.

Although the estimated MW of 56 kD in addition to the poor synthesis of this polypeptide by wheat germ suggested that the 56 kD product is indeed the LSU, confirmation was obtained using the Rubisco antibody against reticulocyte lysate *in vitro* translation products. Figure 21 shows that the LSU can be immunoprecipitated from among 4 d polysomal RNA directed translation products (lane 2) but is not apparent in the lane of the corresponding M (lane 5). The pSSU, on the other hand, is immunoprecipitated from both the polysomal RNA directed products (lane 2, N and lane 5, M) and the poly(A) RNA directed polypeptides (lane 3, N and lane 6, M). Thus the observed deficiency in Rubisco content in the M would appear to be directly related to the lack of translatable LSU mRNA associated with

polyribosomes. Furthermore, as equivalent amounts of TCA-precipitable cpm were used for immunoprecipitation in Figure 21, it is tempting to suggest that a reduced quantity of pSSU mRNA is represented among equivalent amounts of poly(A) RNA isolated from the M as compared to the N. However, this assumption is rather tenuous as many factors can influence cell-free protein synthesis.

Figure 19. Immunoprecipitation of pSSU of Rubisco from wheat germ translation products using poly(A) RNA and total polysomal RNA from 4 d N light-grown seedlings. Lane 2 contains translation products directed by poly(A) RNA and the corresponding immunoprecipitate is in lane 3. Lane 4 was loaded with total polysomal RNA-directed polypeptides and its corresponding immunoprecipitate is in lane 6. Lanes 1 and 4 are N poly(A) RNA and polysomal RNA-directed products synthesized by reticulocyte lysate, respectively, and are included for comparison. Antiserum used against endogenous wheat germ products or the control 0 μg RNA translation assay sample is in lane 7. The fluorogram was exposed for 1 d.

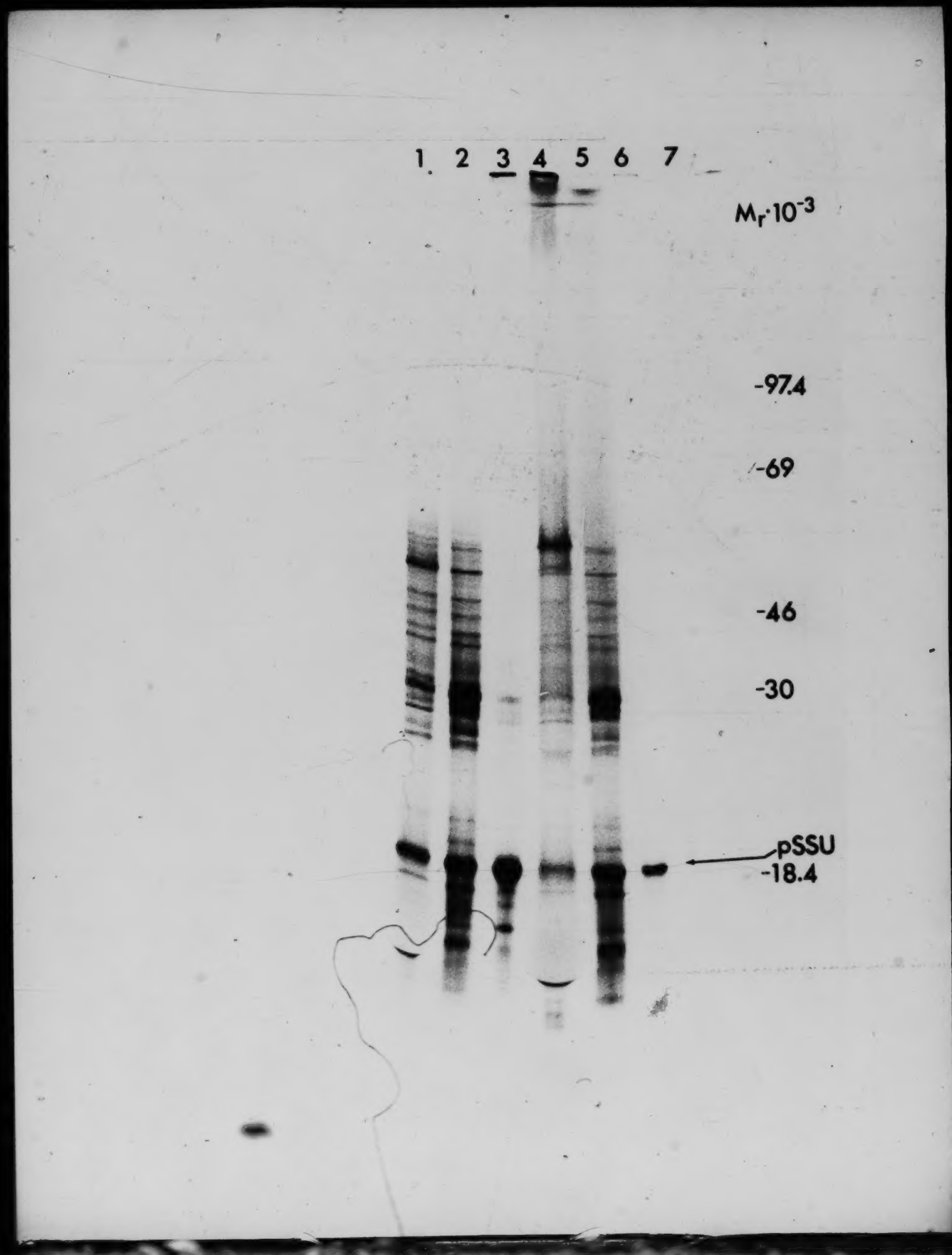

1 2 3 4 5 6 7
$M_r \cdot 10^{-3}$
-97.4
-69
-46
-30
pSSU
-18.4

Figure 20. Immunoprecipitation of pSSU of Rubisco from wheat germ translation products using poly(A) RNA and total polysomal RNA from 4 d M light-grown seedlings. Lane 2 contains translation products directed by poly(A) RNA and the corresponding immunoprecipitate is in lane 3. Lane 4 was loaded with total polysomal RNA-directed polypeptides and its corresponding immunoprecipitate is in lane 6. Lanes 1 and 4 are of M poly(A) RNA and polysomal-RNA directed products synthesized by reticulocyte lysate, respectively, and are included for comparison. The fluorogram was exposed for 5 d.

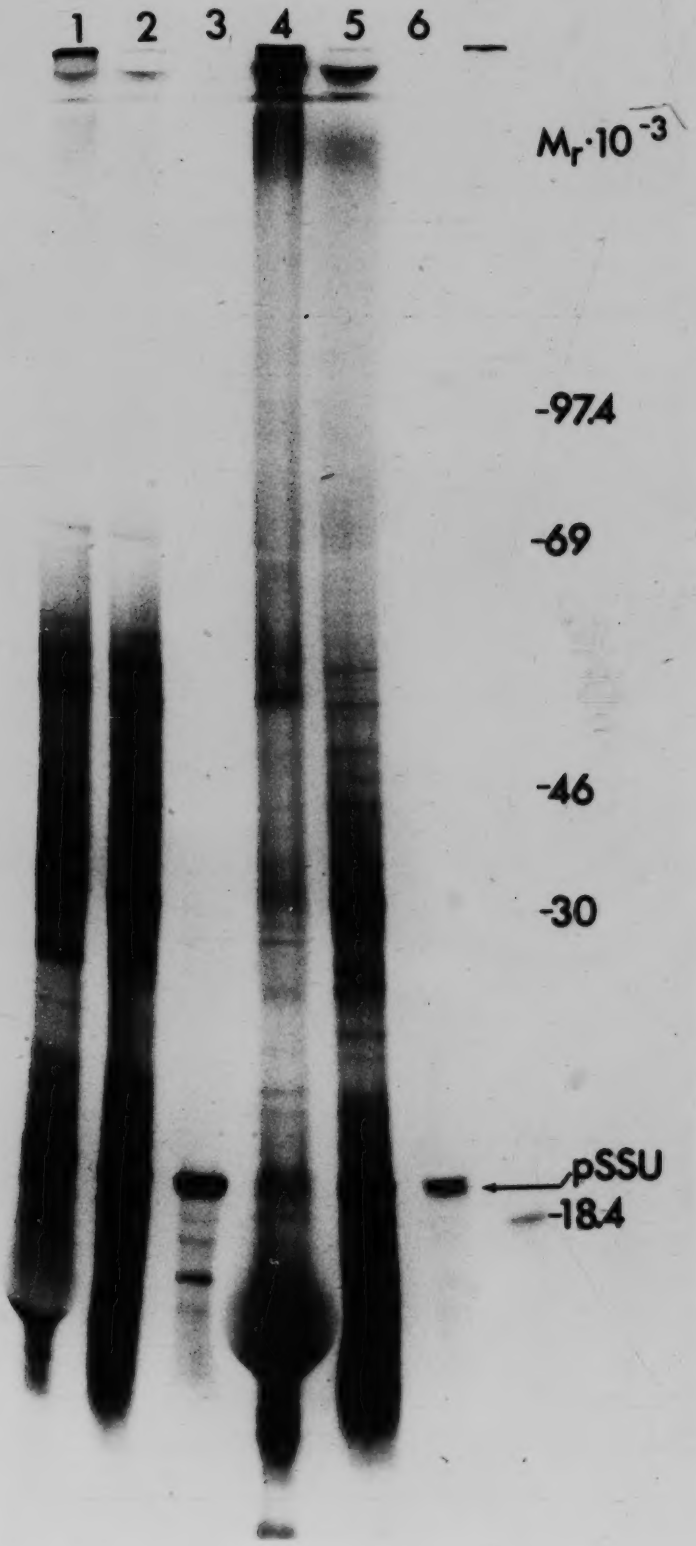
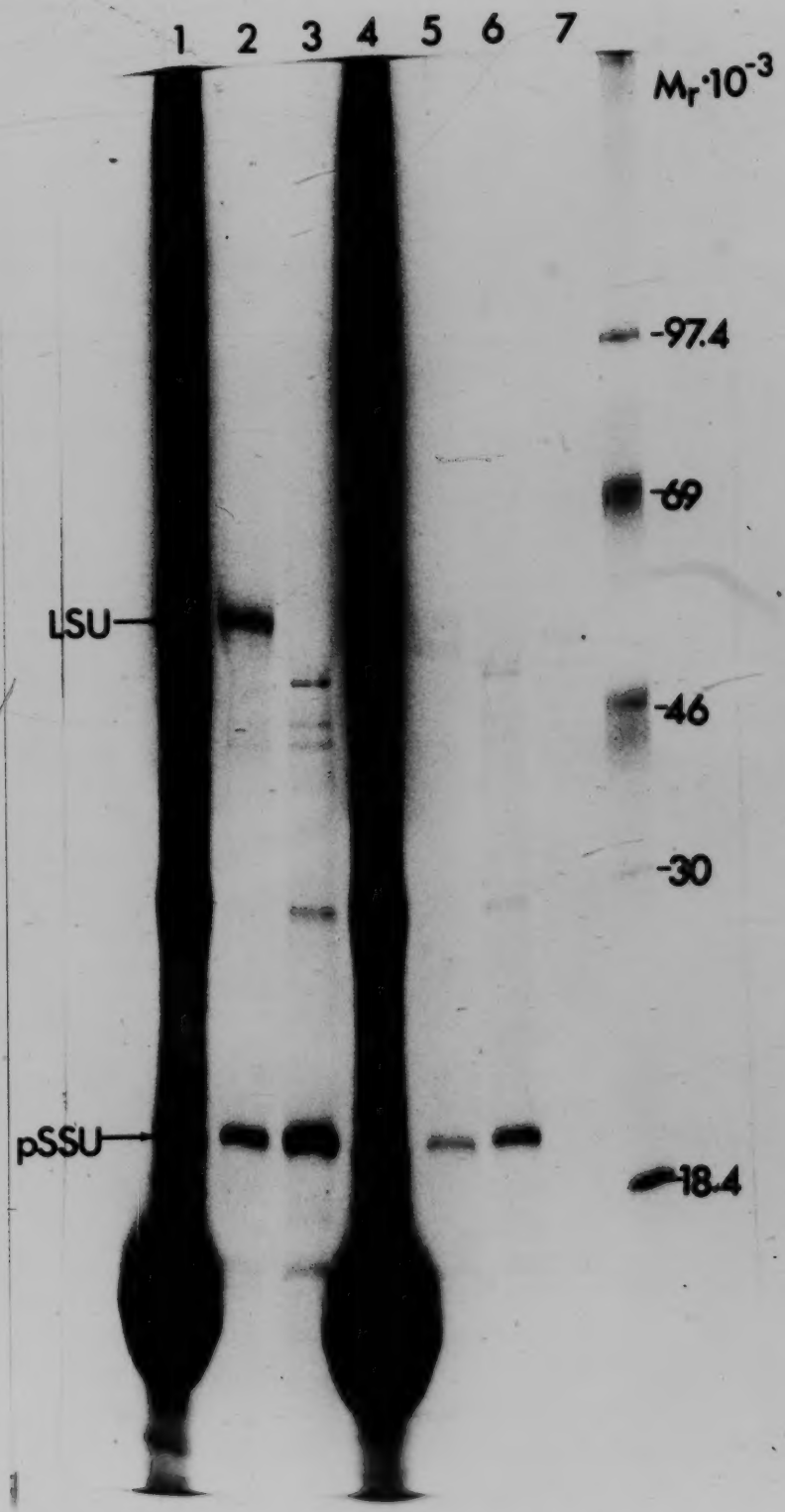

1
2
3
4
5
6
$M_r \cdot 10^{-3}$
-97.4
-69
-46
-30
pSSU
-18.4

Figure 21. Immunoprecipitation of LSU and pSSU of Rubisco from reticulocyte lysate translation products using poly(A) RNA and total polysomal RNA from 4 d N and M light-grown seedlings. Lanes 1 and 4 contain total polysomal RNA products for N and M, respectively. Immunoprecipitates of these products are in lanes 2 (N) and 5 (M). Lanes 3 (N) and 6 (M) are immunoprecipitates of translation products of poly(A) RNA. Antiserum used against endogenous lysate products or control 0 μg RNA translation assay sample in lane 7. Approx. 1,000,000 cpm of [^{35}S]-methionine-labelled protein used for each immunoprecipitation. The fluorogram was exposed for 12 d.


1 2 3 4 5 6 7
$M_r \cdot 10^{-3}$
-97.4
-69
-46
-30
-18.4
LSU
pSSU

V. Conclusions

If one were to assume that the efficiency of isolation of poly(A) RNA from the N and the M was equivalent, Table I shows that the M is at least as well-endowed with poly(A) RNA as the N. When this product is used to direct protein synthesis in a wheat germ system, N and M poly(A) RNA proved to be equally active in directing the incorporation of either [^{3}H]-leucine or[^{35}S]-methionine into TCA-insoluble polypeptides (Table III). Furthermore, the observation that poly(A) RNA isolated from both seedlings could be used to synthesize many discrete polypeptides of varying MW using either wheat germ (Fig. 6) or reticulocyte lysate (Fig. 7) cell-free systems indicates that there is no reason to suspect that mRNA from either source has not undergone the post-transcriptional modifications required to yield a translatable product. Post-transcriptional modifications generally include processes of polyadenylation, methylation, 5' 7-methylguanylate "capping" or processing of precursor transcripts to mature, translatable size. The assumption made here is that neither cell-free system used in this study is capable of recognizing and then carrying out these modifications *in vitro*. Figure 14 shows that mRNA may be isolated from polysomes and translated *in vitro* suggesting that movement of transcribed mRNA from the nucleus to the cytoplasm may also proceed in the M without apparent problem.

Although Figures 5, 6 and 7 indicate that analysis of the *in vitro* translation products by the electrophoretic method used in this study resolved many polypeptides, no obvious aberrant features of the M product profile were noted, particularly among the more abundant messages translated. Perhaps this observation is not entirely unexpected as mRNA provides only a signal of potential polypeptide products and the presence of translatable mRNA cannot be taken as an absolute indication that a physiologically active polypeptide will arise. For example, studies using the chlorophyll b-less mutants of barley (5,19) or pea (180), chlorophyll deficient mutants of pea (180) or maize (135) and chlorophyll b deficient seedlings produced by intermittent illumination (52,216) demonstrate that all of these plants possess readily detectable levels of LHCP mRNA yet fail to accumulate LHCP in thylakoid membranes.

Kyle and Zalik (115) identified the mature LHCP of this *virescens* mutant and that of the wild-type. They assigned an apparent MW of 29 kD to this polypeptide in both sources. They also observed that LHCP was not represented in as abundant quantities among the polypeptides of M plastids as compared to those of the N.

In keeping with the principle of precursor synthesis which has been observed for all of the cytoplasmically-synthesized, chloroplast-destined polypeptides studied to date, a larger than 29 kD pre-LHCP polypeptide would be expected among the poly(A) RNA directed *in vitro* translation products. The MW of all pre-LHCP polypeptides studied to date has been approximately 4 to 5 kD larger than the mature product (150,19). Therefore, analysis of poly(A) RNA directed wheat germ translation products seen in Figure 6 provide a number of candidates for pre-LHCP in the size range of 30 to 34 kD. Bellemare *et al.* (19) reported that the main barley LHCP apoproteins appear as two, closely migrating polypeptides using similar SDS-PAGE gradient gels as those used in this study. Müller *et al.* (151) provide evidence that LHCP mRNA is initiated preferentially in wheat germ in comparison to other poly(A) RNAs or polysomal RNAs. These observations suggest that the two rather abundant, closely migrating polypeptides with an apparent MW of 32 and 33 kD which are synthesized very well by 4 d N and M poly(A) RNA translated *in vitro* using wheat germ (Fig. 6) but not represented well by 4 d poly(A) RNA directed reticulocyte lysate products (Fig. 7), are likely the pre-LHCP components. Identification of LHCP apoproteins remains tentative as antibodies against LHCP were not employed in this study. However, no notable differences between the N and M can be distinguished between the polypeptides of 30 to 34 kD among *in vitro* translation products of 4 to 6 d old light-grown seedlings (Fig. 6 and 7). The apparent failure of LHCP to accumulate in M plastid membranes may well be explained by the reduced levels of chlorophyll a and, perhaps more importantly, chlorophyll b which is especially deficient at 4 and 5 d of development (Fig. 2 and 4).

While stabilization of LHCP by chlorophyll has been implicated as an important point of post-translational regulation of LHCP (6,52,92), a somewhat analogous control mechanism

has been proposed for the appearance of Rubisco. For example, the *Chlamydomonas reinhardtii* ac 20 mutant lacks detectable Rubisco (80,209) yet possesses wild-type levels of pSSU mRNA and at least part of this mRNA is associated with the translational machinery of the cell (144). The lack of Rubisco accumulation would appear to be a consequence of the failure of plastids to synthesize LSU essential for Rubisco assembly. In addition, the report of Silverthorne and Ellis (184) and the research conducted on a nuclear gene mutant of *Petunia hybrida* (50) which employed a cDNA probe to measure levels of LSU mRNA both suggest that changes in the synthesis of LSU reflect fluctuations in the level of translatable mRNA for this subunit.

Taking both of these observations into consideration, it is not surprising that in the case of this M, pSSU can be readily detected and immunopreciptated from the *in vitro* translation products directed by either poly(A) RNA or polysomal mRNA using either wheat germ (Fig. 19 and 20) or reticulocyte lysate (Fig. 21) as the cell-free protein synthesis system. The very low levels of Rubisco reported earlier (12,106), particularly in 4 d M seedlings, can be explained by the failure of plastids to synthesize wild-type levels of LSU. This assessment is supported by the evidence that little LSU mRNA appears to be associated with polysomes at 4 d of development (Fig. 21, lane 5). Any pSSU synthesized in excess of LSU available would, presumably, be proteolytically degraded.

The failure to observe wild-type levels of LSU produced by *in vitro* translation of polysomal RNA could arise either because the LSU mRNA is transcribed at very low levels in M plastids or, alternatively, because 70S ribosomes make only a negligible contribution towards M polysomal profiles at 4 d of development. Unfortunately, neither possibility is mutually exclusive. That is, transcriptional difficulties within the plastid may not only affect the population of mRNA (including LSU mRNA) but might very well affect the levels of tRNA and rRNA available for organellar protein synthesis. Reduced levels of rRNA would lead to an inevitable reduction in 70S ribosomes available for polysome assembly. Even if rRNA were singularly affected, unless LSU mRNA could become associated with 70S polysomes the accumulation of this subunit would most certainly be reduced.

As coupling of the synthesis of pSSU and LSU have been proposed, a slightly reduced amount of translatable pSSU mRNA in the M is not unexpected. This somewhat reduced quantity of pSSU represented among 4 d M *in vitro* translation products is suggested by poly(A) RNA directed products of wheat germ (Fig. 19 and 20, lanes 3) and reticulocyte lysate (Fig. 21, lanes 3 and 7). Furthermore, immunoprecipitation of pSSU synthesized by a reticulocyte lysate cell-free system shown in Figure 21 supports this observation. However, Figure 21 also provides evidence that M polysomes synthesize pSSU despite the poor translation of LSU, suggesting that the synthesis of these two subunits is not tightly coupled in the M. A disadvantage realized by *in vitro* translation and subsequent immunoprecipitation is that quantification of messages is not possible nor is this approach as sensitive or as conclusive as results provided by RNA-DNA hybridization studies. While a cDNA probe for pSSU mRNA would be useful in measuring the magnitude of the difference in quantities of this message present in the N and M, the question of whether or not transcription of rbcL proceeds at wild-type levels would best be addressed using a suitable cDNA probe for LSU mRNA.

Although the very distinct pale-green phenotype of light-grown M seedlings provides a striking contrast to the N, the mutation is expressed even in dark-grown plants which are phenotypically very similar. Distinct features of etiolated M seedlings include reduced levels of protochlorophyllide (127), abnormal etioplast ultrastructure (106) and a quantitative difference found between polysomal profiles of etiolated N and M tissue shown in Figures 10 and 11. Polysomal profiles of 4 d etiolated M seedlings are distinguished by the lack of a peak found in comparable N profiles. This peak, as described in "Results and Discussion", is very likely to be identified with 70S ribosomes.

Unfortunately, a number of possibilities could be entertained in attempting to explain the apparent reduction in 70S ribosomes. Research published by Mets and Bogorad (139) demonstrated that mutations can arise in either the cytoplasmic or organellar-synthesized components of ribosomes. Conceivably, such mutations could lead to 70S ribosome assembly problems. Low carotenoid levels have been implicated in causing the premature

photo-destruction of newly formed 70S ribosomes in the *tigrina* o^{34} barley mutant (99). Another possibility that has been explored is that the activity of chloroplast DNA dependent RNA polymerase may be a primary cause in reducing 70S ribosomes by preventing the transcription of plastid rRNA. However, results using heat-treated 70S ribosome-less seedlings (33) and the plastid ribosome deficient *albostrians* mutant of barley (183) show that RNA polymerase activity is not markedly affected in either of these plants. These results do serve to substantiate the nuclear location of synthesis for this enzyme as originally proposed using inhibitor studies (65). Due to the complicated interdependence between the activities of the nuclear and plastid genomes, as seen even in rates of plastid transcription, the actual cause of the absence 70S ribosomes, either by physiological heat-treatment or by mutation remains an enigma.

In the case of this *virescens* mutant, it is difficult to resolve whether or not the reduced quantity of 70S ribosomes is an additional symptom of the nuclear mutation or a direct consequence of the lesion. Furthermore, as 70S ribosomes are required for the synthesis of many of their ribosomal proteins, a reduced protein-synthesizing capacity of plastids would be accentuated if the demand for more plastid products arose, which might very well occur during early stages of chloroplast biogenesis.

A previous study analyzing ribosomal components of the N and M barley (113) led to the suggestion that the M may not be as competent as the N in terms of cytoplasmic translational capacity. However, comparison of 4 d N and M polysomal profiles (Fig. 8) suggests that polyribosome formation in the M is apparently unaffected. However, it is not advisable to extrapolate *in vivo* rates of protein synthesis from such profiles without considering estimates of *in vivo* rates of translation initiation, elongation and termination (55). These estimates are not accounted for by the simple rates or percentages expressing polysomal aggrégation presented in Table IV. Perhaps one of the very few detailed reports with respect to estimates of *in vivo* protein synthesis was published by Baumgartel and Howell (15) using *Chlamydomonas reinhardtii*. These authors were able to estimate rates of translation initiation

and elongation by pulse labelling the nascent polypeptide chains associated with polysomes. In this manner the authors were able to obtain more reliable estimates of the *in vivo* rate of protein synthesis than those determined by measuring the uptake and incorporation of a radioactive amino acid. This report showed that polysome aggregation in this organism is largely a function of rates of polypeptide initiation. While initiation is a primary regulator of translation, rates of elongation were found to play only a minor role. However, as *Chlamydomonas reinhardtii* provides a rather advantageous organism to conduct pulse-labelling experiments, it is not surprising that few studies of this nature have been attempted for higher plants.

Mösinger and Schopfer (148) were able to show that exposure of dark-grown seedlings to light resulted in a considerable aggregation of polysomes accompanied by the concomittant recruitment of newly transcribed mRNA. By comparing the profiles of 4 d etiolated seedlings (Fig. 10) to those of light grown material (Fig. 8), it is evident that the response of the N to light, as seen by the aggregation of polysomes, is also featured in M profiles. Furthermore, extraction and *in vitro* translation of polysomal RNA demonstrates that translatable mRNA is indeed associated with ribosomes in the M and the N. However, there is no unequivocable evidence to contend that the actual rates of *in vivo* protein synthesis are equal or, in fact, different between N and M seedlings. Perhaps the most judicious interpretation of the polysomal profiles depicted in Figures 8 to 11 and the ratios tabulated in Table IV is that the M responds to light by enhanced polysome aggregation and it would appear that the M is well-equipped to conduct cytoplasmic protein synthesis. However, the protein synthesizing capacity appears to be reduced in young, M seedlings by a decreased complement of 70S ribosomes.

A possibility that should be raised is that the import of metabolites across the M plastid membranes may be impaired. Metabolites that are transported across the chloroplast membrane include amino acids, sugars and polypeptides. The recent report of two mitochondrial protein transport mutants of yeast (236) suggests that conditional mutants of this nature could also give rise to pleiotropic effects. The temporal recovery made by this mutant would be construed

as a "funneling" effect with the accumulation of building materials within the chloroplast being instrumental towards the eventual recovery of chloroplast function. However, there is no specific evidence presented by this study that would support or, indeed, exclude this explanation. Evaluation of plastid envelope transport functions is complicated by the difficulties encountered in purifying intact barley plastids.

An observation raised in the earlier part of this discussion is that *in vitro* translation of N and M poly(A) RNA did not reveal any qualitative differences between the products synthesized. However, concerns have already been addressed in the "Results and Discussion" regarding the reliability of *in vitro* systems in responding to exogenously added messages. The preferential initiation of certain messages such as LHCP mRNA (151) serves to underscore the caution that should be exercised in making quantitative judgements from wheat germ generated polypeptide profiles. However, it is hoped that any factors which would have affected *in vitro* translation of N products would have similarly affected those of the M. Furthermore, for the wheat germ assay system, comparisons made for any particular product across lanes might be safer than quantitative evaluations made for different polypeptides within any given lane.

The selective nature of the wheat germ system is shown by the apparent inability of this system to synthesize LSU as shown by comparing lanes 4 and 5 in Figure 19. Also, while LSU may be readily identified and immunoprecipitated from among reticulocyte lysate products (Fig. 21, lane 2) no comparable immunoprecipitate is detected from wheat germ products directed by a similar source of RNA (Fig. 19, lane 6). The fact that both systems are eukaryotic in nature excludes the argument of difficulties engendered by prokaryotic-eukaryotic translation barriers as originally proposed by Bottomley *et al.* (26). More recently, Lerbs *et al.* (123) provide evidence that some batches of wheat germ can be used to successfully translate chloroplast mRNA from spinach, *Nicotiana* and *Euglena* but only in the absence of cytoplasmic mRNA. They report that even minor levels of cytoplasmic mRNA appear to suppress the synthesis of chloroplast mRNA-encoded products. These observations help to reconcile the differing and rather contradictory opinions expressed about the value of wheat

germ for chloroplast and cytoplasmic mRNA studies (48,162).

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